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WINTER SCHOOL

ON

Recent Advances in Mariculture Genetics and Biotechnology

4th to 24th November 2003

Course Manual

INDIAN COUNCIL OF AGRICULTURAL RESEARCH
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PREFACE

Food security will be a major issue facing the mankind in near future. The challenge for the agricultural sector is to double food production by year 2025 whereas in case of seafood production, it need to be increased approximately sevenfold. This calls for enhancing the production from marine and inland aquaculture, which at present is mostly low tech. Application of genetics and biotechnology can play a major role to accelerate the development and increase the output. The Central Marine Fisheries Research Institute, established in 1947 is the nodal agency in India responsible for research support in marine fisheries development. The Institute has won the recognition as a premier Institute comparable to any other well-established marine fishery institute in the world. The Institute has, over the years, built up laboratories and field facilities for carrying out research in marine fisheries and mariculture. The Institute caters to the research needs of marine fishery resources assessment and management, development of mariculture practices and investigations in the frontier areas of mariculture nutrition, physiology, pathology, genetics and biotechnology.

In recent years several advance techniques have been developed in the area of genetics and biotechnology which can have application in aquaculture. The winter school on "Recent Advances in Mariculture Genetics and Biotechnology" being conducted at CMFRI is sponsored by ICAR and aims to cater to the needs of young research scientists and teachers in the field of Genetics and Biotechnology and bring about qualitative improvement in their knowledge and skills in some of the important platform technologies in Mariculture Genetics and Biotechnology.

The manual being released on this occasion contains the lectures presented by the faculty. I have great pleasure to record my whole-hearted appreciation to all the faculty members and guest teachers for their sincere and dedicated work. Prof. Dr. Mohan Joseph Modayil , Director CMFRI has extended all the possible cooperation and guidance in organizing the Winter School for which I am grateful to him. I also thank the coordinators of the Winter School for their continued support in looking after the various academic as well as other programmes. I also extend my gratefulness to all other colleagues for making arrangements for the conduct of the practical sessions.

I also express my gratitude to the Indian Council of Agricultural Research for sponsoring the Winter School and funding this manual. I am sure this manual would enable the participants to enhance their knowledge and competence in the field of Mariculture Genetics and Biotechnology.

3rd November 2003
Kochi-14

Dr.P.C.Thomas
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PERSPECTIVES IN MARINE BIOTECHNOLOGY

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Introduction

The Convention on Biological Diversity (CBD) defines Biotechnology as: "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use". According to Dr. Rita Colwell, Director, National Science Foundation, USA, the apt definition is " making money from Biology". Biotechnology involves application of scientific and engineering principles to provide goods and services through mediation of biological agents. Traditional application includes antibiotic production through fermentation, microbial sewage treatment and water purification. Modern molecular biotechnology involves gene manipulation and gene transfer, DNA typing, bacterial cloning for production of biomolecules etc.

In 1992, an Organization of Economic Co-operation and Development report on biotechnology defined marine biotechnology as "the application of scientific and engineering principles to the processing of materials by marine biological agents to provide goods and services." This would include biotechnology-based methods for aquaculture, fisheries, and marine natural products. Marine biotechnology is gaining momentum due to its vast potential application in augmenting seafood production, safeguarding human health, protecting and restoring aquatic environment (bioremediation), reducing fouling and corrosion, exploiting marine natural products for human benefit, conserving biodiversity, managing fish and shellfish resources and in clarifying fish and shellfish species status.

The gene pool and biological processes of large majority of marine organisms including microorganisms are not known well enough. However, exploratory research shows the potential for exploiting the biochemical capabilities of marine organism to provide models for new classes of pharmaceuticals, polymers, enzymes, other chemical products, industrial processes as well as vaccines, diagnostic and analytical reagents, genetically altered organisms for aquaculture and seafood industry.

Biotechnological Interventions in Aquaculture:

Aquaculture is receiving importance as extreme focus sector for development in view of its role in augmenting production of nutritious food besides a large variety of natural products. Recent researches have amply proved the need for biotechnological interventions in sustaining aquaculture production. Significant growth enhancement has been achieved through hormonal intervention and gene manipulation. Success stories are documented on biotechnological interventions in induction and control of maturation and spawning, sex

control (gynogenesis and androgenesis), sex inversion in protandrous species like sea bass and protogynous species like the grouper, genetic improvement (production of triploids and tetraploids and transgenic species), in disease diagnosis (molecular and immunodiagnostic kits) and management (probiotics, vaccines, immunostimulants, gene therapy), cell and tissue culture (in vitro pearl production), conservation of germ plasm (cryopreservation of gametes and embryos)

Biotechnological interventions in aquafeed production include bioencapsulation of live feed, nutrient enrichment of raw materials (fermentation), reducing antinutrients and crude fibre levels, production of enzymes - α-alpha amylase, β-amylase, cellulase / hemi-cellulase & phytase) amino acids (l-lysine, threonine & monosodium glutamate), microbial polysaccharides, vitamins (B 2, B 12, Biotin), organic acids (lactic acid), flavours, growth promoters, carotenoids (Ha*ematococcus, Dunaliella),* essential fatty acids (bacteria, fungi & *thraustochytrids)*

Genomic and genetic improvement

The main thrust area in genetic improvement of farmed fish and shellfish is to enhance desirable traits (phenotypic character) of high economic importance – growth rate, size at first maturity, egg number, egg size, egg weight, egg survival, larval survival, disease resistance, behaviour, resistance to environmental factors, dressing weight, carcass quality, fat content, protein content, food conversion, anatomic modification, colour and others.

Genomics will help identify and select with precision useful genes in fish, invertebrates, and algae for aquaculture. Genome mapping is being carried out in a number of economically important aquaculture species.

Production of transgenic fish by inserting desirable genes through electroporation and microinjection has been successfully carried out since the mid-1980s. In a number of cases, fish growth hormone genes, along with a reporter gene, which will tell whether the transformation is successful, are inserted into the fish. There are reports of better growth in transgenic tilapia, catfish, and rainbow trout than non-transgenic individuals.

The primary goal of genomics is to identify genetic diversity within stocks and identify genes that can be transferred into fish or invertebrate eggs and result in transgenic animals with improved safety and economic benefit such as better and faster growth rates, disease resistance, greater toleration for environmental change (e.g., cold water tolerance), increased fecundity, and more edible meat and to produce pharmaceuticals.

Biotechnology methods have resulted in production of new, genetically engineered vaccines for aquaculture, such as the vaccine against infectious hematopoietic necrosis (IHN) virus, which is responsible for the death of trout and other salmonid stock.

Bioactive compounds and biomolecules

The marine bioactive compounds or marine natural products (MNPs) offer avenues for developing cost-effective, safe and potent new drugs and other useful products. MNPs are organic compounds produced by microbes, sponges, seaweeds, and other marine organisms. The host organism biosynthesizes these compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment. In the decade from 1977 to 1987, around 2500 new metabolites (MNPs) were reported from marine organisms ranging from microbes to fish. . Perusal of literature indicates that even the seawater has bactericidal properties, which is primarily attributed to the production of antibiotics by planktonic algae and bacteria respectively. Researches carried out globally resulted in identification of hundreds of free-living and symbiotic marine microorganisms and numerous marine invertebrates that have the potential to produce new pharmaceutical compounds cosmetics and neutraceuticals.

A large variety of marine micro and macro organisms have been screened and some of them are found to contain anti-viral, anti-microbial and anti-inflammatory compounds. Micro algae are rich sources of bioactive compounds. Cyanobacteria contain natural compounds that also serve as models for drug development. By determining metabolic control mechanisms and restrictions, as well as optimizing culture media for production of secondary metabolites and methods for the isolation and identification of genes controlling secondary metabolism could help in large scale culture of important species.

Bugula neritina, a slow growing marine bryozoan is the source of a potential drug, which is active against leukemia. Prostaglandins isolated from marine organisms like gorgonids are found to be active against chemotherapeutically resistant ovarian cancer. Anti-inflammatory drugs could also be isolated from marine algae, by developing proper culture techniques.

Marine organisms, many of which can be mass cultured, are sources of commercial products, among which are food additives such as carrageenan, agar, and chitin; commercial glues; enzymes for detergents and for biological reagents; and other products.

Nutraceuticals, such as food-based enhancers, and cosmetics also can be produced from marine sources. The soft coral, *Pseudopterogorgia elisabethae*, is the source of pseudopterosin that is used as a cosmetic additive.

In sponges, the secondary metabolites are synthesized to protect themselves and to maintain homeostasis. The wider biosynthetic capability of sponges could be attributed to their biological association with other symbionts. A wide variety of secondary metabolites were isolated from sponges and these have been associated with antibacterial,

antimicrobial, antiviral, antifouling, HIVprotease inhibitory, HIV reverse transcriptase inhibitory, immuno- suppressent and cytotoxic activities. In addition to potential anticancer applications, the MNPs of sponges have a myriad of activities ranging from antibiotic activity including anticoagulant, antithrombin, anti-inflammatory, as well as imunomodulatory activities.

Many marine organisms have also been documented to be sources of prostaglandins-PG1, PG2 , and PG3, and fatty acids like docosahexaenoic, eicosapentaenoic and arachidonic acids, which are receiving importance in human and animal nutrition and health care. The sea is also the richest source of carotenoids – beta carotene, astaxanthin.

A thermo stable alpha-galactosidase, which can hydrolyse melibiose oligomers in soya and other bean products, is isolated from the bacterium, *Thermotaga neaopolitana* from oceanic blacksmokers. Enzymes extracted from *Archaea thermococcales* (DNA polymerase having 5 times more half life) will increase efficiency in biotechnological process requiring replication of DNA. Phytases are produced by a variety of bacteria and fungii.

New mineral organic materials could be developed from certain marine organisms, which consist of diatom produced biosilicate and chitin fibres. These materials are biodegradable and exhibit a remarkably high strength to weight ratio and are superior to synthetic materials used in industry. Industrial surfactants could be manufactured from fishmeal processing wastes.

Environmental bioremediation

Microbes have been successfully used in bioconversion for waste management in controlling pollution, detoxification of industrial organics such a PCBs, PAHs, and creosote, and heavy metals copper, chromium and arsenic to harmless products. Transgenic Cyanobacterium, *Synechococcus*, with the gene for mammalian metallothionein, increase its heavy metal resistance and accumulation

Cyanobacteria Synechococcus and green alga Chlamydomonas *reihardtu* have been effectively used against heavy metal (cadmium) contamination by its genetic modification by inserting a gene for increasing its heavy metal resistance and accumulation.

Solving fishery related problems

Biotechnological tools can be applied in stock assessment of fish and shellfish, for studying genetic diversity of different marine organisms; ratification of taxonomic status of fish and shellfish and for identification of distinct stocks of fish and shellfish species and the degree to which they inter-mix

Biosafety and environmental issues

Biosafety concerns raised against commercial production of transgenic fish or shellfish must be considered on a case-by-case basis, focusing on the species, culture system, and ecosystem and quality of the products derived. Other major concerns are possible impact if the transgenic animals escape into the environment and the parental stocks that are genetically modified but not sterile.

Conclusion

Marine Biotechnology is just beginning to revolutionize our ability to better use marine resources... In the US 190 patents were granted from 1983 to 1995, 30 marine products targeting cancer, inflammation and AIDs; the market value of 5 of the products is valued at 2 billion US$. One anti-inflammatory agent P*seudoterosin* derived from soft corals has projected sales of 100 million US$. Japan has set up a network of Marine Biotechnology Laboratories investing more than 1 billion US $ to harness the enormous potential. In India Shanta Marine Biotech and EID Parry have already set up industries to produce astaxanthin from *Haematococcus* and beta carotene from *Dunaliella*.

In the future advances in such areas as bioconversion, biomaterials, pharmaceuticals and diagnostics, aquaculture, seafood safety, bioremediation, and biofilms and corrosion would play vital roles in sustainable development programmes. Currently a multibillion dollars industry worldwide we can expect greater leaps in Marine Biotechnology in the future

AN OVERVIEW OF THE GENETIC TECHNIQUES FOR IMPROVEMENT OF FISH AND SHELLFISH FOR AQUACULTURE

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Introduction

There are two ways in which an aquaculturist can increase his yields. The first is by environmental manipulations, such as the optimal use of fertilizers, feeds, improved water quality management etc. The second is by growing genetically improved stock. Any of the different genetic manipulation techniques available can be employed for producing genetically improved brood stock. In addition to the conventional quantitative genetic techniques like selection and breeding, modern tools like chromosomal engineering for production of polyploids, gynogenesis and androgenesis can also be employed. The most modern technique is genetic engineering where in a desirable gene or set of genes from any source can be transferred into a fish for producing a transgenic fish with desired characteristics. However, an in-depth knowledge of the science of fish genetics is a pre-requisite for the formulation of the appropriate techniques for their improvement.

Quantitative genetic tools

Breeding is the applied science of genetics. There are a number of selection and breeding programmes that can be used for the genetic improvement of fish. These are the two traditional techniques that have been used for thousands of years to improve all major crops and livestock grown by farmers. Although, farmers have conducted scientific breeding programmes on livestock for thousands of years, fish farmers are only beginning to use selection, hybridisation, or other breeding programmes to improve aquaculture species of food fish. Although some progress has been made, many fish farmers are culturing fish that are essentially wild and unimproved.

The ultimate goal of every selective breeding programme is to improve the breeding value of the population. The fish genes determine the breeding value. A farmer hopes that when he improves the breeding value of his population its monetary value also will be improved, which is determined by the fish's phenotypes. To accomplish this goal, a breeder selects (saves) fish that possess certain desired phenotypes and culls (removes) those that do not. By selecting and mating only the best fish (largest, heaviest, those with the desired colour, etc.) he hopes that the selected brood fish will be able to transmit their superiority to their offspring, thereby creating a genetically improved population. If this occurs, the next generation will be more valuable because the fish will grow faster, which will increase yields; the fish will grow more efficiently, which will lower feed costs; or all fish will have a more

desired body colour, which will increase their market value. There are a number of methods, which can be used for selection of superior genotypes for scientific breeding.

Inbreeding and crossbreeding (hybridisation) are the two traditional breeding approaches that have been successfully used for the improvement of crops and livestock. Inbreeding is often combined with hybridisation to improve the results of the crossbreeding programme. Crossbreeding is a breeding programme that tries to find mating combinations between different populations of fish which produce superior offspring for grow-out, offspring that are said to exhibit hybrid vigour. Although crossbreeding is a tried-and-true method of increasing yields, the results of crossbreeding programmes are impossible to be predicted (unless the mating has been made previously), so the production of superior offspring is a hit-or-miss proposition. Many combinations often have to be evaluated before a combination that produces offspring with hybrid vigour is discovered. Crossbreeding programmes usually involve different strains within a species (intraspecific hybridization), but different species can also be hybridised (interspecific hybridisation). To date, much of the breeding work in aquaculture has been devoted to hybridisation among the different species of tilapia in an attempt to produce all hybrids for grow-out.

In general, crossbreeding is used to produce superior fish for grow-out, while selection is used to create superior brood fish. The hybrids that are created in a crossbreeding programme are usually grown and sold as food. A farmer rarely retains and spawns the hybrids to produce a new generation of production fish. On the other hand, brood fish that are created in a selective breeding programme are created for one purpose – to produce the next generation of genetically superior fish for grow-out and their offspring can, in turn, be retained and selected to continue the process of producing next generation of improved stock.

In recent years, cytogenetic research had led to the development of three additional breeding programmes that can be used to increase yields. One of the most common breeding programmes in aquaculture is the production of sex-reversed brood stock to produce monosex populations for grow-out. This is done either because one sex is superior or more desirable or to prevent reproduction during grow-out. For example female sturgeon are more valuable than males because they are heavier; female salmons are the more valuable sex because sexually precocious males die before they can be harvested; however male tilapia are more desired than females because they grow twice as fast. The major goal in tilapia farming is to prevent reproduction during grow-out; this can be best accomplished by producing a monosex male population.

The production of sex-reversed brood stock is usually accomplished by feeding sex hormones either estrogens or androgens to sexually undifferentiated fry to sex-reverse them. Sex-reversed fish are individuals that are one sex phenotypically but the other genetically. If sex reversal is done properly, sex-reversed fish are capable of producing monosex populations for grow-out/ The type of hormone used- estrogens to produce sex-

reversed females or androgens to produce sex-reversed males-depends on the sex-determining system of the species and whether you want to produce an all male or an all female population.

Chromosomal engineering

Another programme that is becoming more commonplace is the application of chromosomal manipulation. The most common form of chromosomal manipulation is the production of triploids. This involves the use of temperature or pressure or chemicals to shock newly fertilized eggs. If shock is applied properly, it prevents the second polar body from leaving the egg and therefore, the newly fertilized egg contains a haploid sperm nucleus, a haploid egg nucleus, and a haploid second polar body nucleus. These three haploid nuclei fuse and produce a triploid zygote, which in turn, produces a triploid fish. Triploids are sterile. This type of breeding programme is used to enable farmers to grow exotic species whose culture might otherwise be illegal or to induce sterility in species that become sexually mature before they reach market size. For example, grass carp culture in most of the United States is legal only if a farmer raised triploids. This technique can also be used to improve the results of interspecific hybridization.

Chromosomal manipulation can be used to produce animals with genetic contribution from only the mother (gynogens) other only from the father (androgens). This is done by creating haploid zygotes and by then shocking the zygotes to produce diploid zygotes. Haploid zygotes are produced in one of two ways: a normal egg is fertilized by sperm is used to fertilize an egg whose DNA has been destroyed by UV irradiation (gynogenesis); a normal sperm is used to fertilize an egg whose DNA has been destroyed by UV irradiation (androgenesis). Gynogenesis and androgenesis are techniques that can be used to produce highly inbred lines for breeding purposes. It can also be used to produce super males; such males are capable of producing all-male populations.

Genetic engineering

In recent years, a new, high-tech breeding programme has been developed viz. genetic engineering. This is a breeding programme that transfers a single gene or a set of genes from one individual into another. This transfer can be within a species, between two species, or even across kingdoms. Although genetic engineering has generated lots of publicity, there are only very few cases of successful production of genetically superior fish for farmers. Furthermore, this type of breeding programme is very expensive, highly regulated, and requires highly trained scientists. Scientists working at universities, governmental research stations, can generally conduct this type of breeding programme or at agribusinesses that are capable of supporting expensive research projects with secure containment facilities.

Though selective breeding is the simplest of all, the decision to conduct a selective breeding programme is a decision that must be made for each farmer or each fry/fingerling

production centre on a case-by-case basis. Because selective breeding programmes require dedication, a certain level of sophistication, record keeping, and the investment of extra labour. Additionally, selective breeding programmes are not free; they also require the investment of money. Finally, these programmes usually do not produce immediate improvements. Improvements are usually not seen for at least one growing season, so a farmer must be able to incorporate long-term planning into his farm management programme, and he must be patient. As a result, within a region, only a small percentage of farmers or fingerling production centres should or will ever conduct selective breeding programmes. A final requirement that must be met before a farmer can conduct a selective breeding programme is the existence of proper facilities.

When the uncontrolled reproduction resulting significant suppression of yield where the product is not economically marketable, farmers can benefit from breeding programmes that can produce monosex populations or sterile populations. For example, the biggest problem in tilapia culture is the fact that tilapia become sexually mature before they reach market size and, as a result, reproduces in the grow-out ponds. This uncontrolled reproduction means that a significant percentage of yield is unmarketable. Tilapia farmers may benefit from breeding programmes that can produce monosex male populations far more than from selective breeding programmes that might improve growth rate.

Suggested Reading

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Technical paper-3

GENES AND GENOMES: A POST-GENOMIC PERSPECTIVE

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Introduction

Living cells probably arose on earth about 3.5 billion years ago by spontaneous reactions between molecules in an environment that was far from chemical equilibrium. From our knowledge of present-day organisms and the molecules they contain, it seems likely that the development of the directly autocatalytic mechanisms fundamental to the living systems began with the evolution of families of RNA molecules that could catalyze their own replication. With time one of these families of cooperating RNA catalysts developed the ability to direct synthesis of polypeptides. Finally, as the accumulation of additional protein catalysts allowed more efficient and complex cells to evolve, the DNA double helix replaced RNA as a more stable molecule for storing the increased amounts of genetic information required by such cells. Thus all present-day cells use DNA as their hereditary material.

Evolution of genomes

A complete set of the genetic material of an organism is called a genome. Except in the case of RNA viruses, DNA is the genetic material of all genomes. The genomes of plasmids, mitochondria, viruses etc., which possess self-replicating ability, are very small, compact and almost entirely utilized for their life activities. Bacterial genomes are relatively large and are not as economically utilized as in the case of plasmids or viruses. In general the DNA content of genomes increases with evolutionary complexity. There are some exceptions to this general rule, which is seen in the case of flowering plants and amphibians. For many years the lack of precise correlation between the complexity of an organism and the size of its genome was looked on as a bit of a puzzle, the so-called C-value paradox (C representing the amount of DNA in a haploid genome). In fact the answer is simple: space is saved in the genomes of less complex organisms because the genes are more closely packed together. The sizes and the number of genes of a few genomes are given in table1.

Table 1. The sizes and the number of genes of genomes

Genome	Group	Size of genome (Kb)	Appro. Number of genes
Kalilo	In *Neurospora*, a fungus	9	2
F plasmid	In *E.coli*	100	29
Adenovirus	Animal virus	21	50
HCMV (herpes virus)	Human virus	229	200
T4	Bacterial virus	172	300
Homo sapiens mitochondria	Human	16	37
S. cerevisiae mitochondria	Yeast	78	34
M.polymorpha chloroplast	Liverwort	121	136
Hemophilus influenzae	Bacterium	1,830	1,703
Methanococcus jannaschii	Bacterium	1,660	1,738
Escherichia coli	Bacterium	4,700	4,000
Saccharomyces cerevisiae	Yeast	14,000	6,000
Caenorhabditis elegans	Nematode	95,000	13,500
Arabidopsis thaliana	Plant	120,000	25,000
Drosophila melanogaster	Fruit fly	140,000	15,000
Xenopus laevis	Toad	3000,000	25,000
Mus musculatus	Mouse	3000,000	30,000
Homo sapiens	Human	3300,000	35,000
Zea mays	Maize	5000,000	35,000

Self-replicating molecules undergo natural selection

Natural sequence variation is a fundamental property of all genomes. For example, any two haploid human genomes show multiple sites of polymorphisms. Some of these have functional implications, whereas many probably do not. A wide variety of polymorphic loci seen in human chromosomes have been used to investigate the extent of human genetic diversity and to delineate the relationships between modern human populations (Tishkoff *et al.*, 1996). In addition to mitochondrial variation, nuclear loci used include: classical blood group and serological markers, RFLPs, microsatellites,

retrotransposon insertions and haplotypes of closely linked polymorphisms (Armour *et al.*, 1993). When different alleles exist in different populations, additional information on population relationships can be obtained if cladistic information can be used to define the evolutionary relationship among the alleles. For example, the analysis of haplotypes of closely linked polymorphisms has allowed the inference of phylogenetic relationships between the different states encountered and thus the reconstruction of a probable history for that chromosomal segment, in which different haplotypic states differ either by new mutation or recombination.

Evolutionary relationships can be deduced by comparing DNA sequences

Simple tandemly repeated sequences, or microsatellites, are ubiquitous in the genomes of a wide range of organisms, and the number of repeats within many of them is highly variable in the population of a particular species. The advent of the polymerase chain reaction provided the means for rapid and cost effective analysis of the repeat number, and several groups showed that these sequences were likely to provide a rich source of very informative markers for genotyping (Armour *et al.*, 1996; Hauswirth, 1994). By virtue of their extensive length variation between different alleles, they have found many applications in genetic analysis, including the establishment of individual identity and family relationships, parental diagnosis and other forensic analysis. The high level of population variability at these polymorphic loci are due to a high rate of germline mutation to new allelic states, at frequencies (up to 15 % per gamete) high enough to measure by direct observation in pedigrees and single molecule analysis of germline DNA (Jeffreys *et al.*, 1988).

An analysis of this sequence variation allows us to construct the human origins and history. They address questions such as when and where our species first emerged, how our ancestors spread over the globe and what the major events occurred in their history. In particular several data sets indicate that variation of the human gene pool originated in Africa within the last 200,000 years (Armour *et al.*, 1996; Cann *et al.*, 1987). Furthermore, the study of DNA sequences allows the defection or expansions of population size. Using the same techniques it is possible to reconstruct the history of population, their origin and relationship precisely and unambiguously as in the case of parental diagnosis and forensic analysis. Information obtained on the genotyping can be used in resolving ambiguities in evolutionary relationship based currently on analysis of other methods such as palaentological, archaeological, anthropological and radiocarbon studies (see, Thurston and Rangachari, 1975).

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DNA MARKERS FOR FISHERIES APPLICATIONS

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Introduction

Over the past few years, application of molecular genetic markers in fisheries has increased dramatically due to the advances in DNA sequencing, data analysis and PCR and it has been feasible to tackle several issues including population genetics, broodstock development, fish health management, transgenics, genetic diversity, conservation and genomics. Molecular markers are polymorphic DNA or protein sequences that can be used to identify a chromosomal region. The molecular markers blended with the PCR technology have become the central tool in many areas of fisheries research. Excellent reviews on the role of molecular genetic markers in fisheries are available (Carvalho and Pitcher, 1994; O'Reilly and Wright, 1995; Ferguson and Danzmann, 1998). Molecular markers are typically unaffected by environmental conditions unlike meristic and morphometric characters. There are two types of markers based on their origin: *Protein markers* and *DNA markers* and the present paper deals with the latter.

Advantages of DNA markers over protein markers

DNA markers are based on the polymorphisms detected at the DNA level. Polymorph DNA markers serve as landmarks or anchor loci for identification and analysis of new loci/genes in the genome. The methods involving direct examination of DNA are strikingly different from allozyme techniques in the following respects (a) the range of potential genetic markers that can be assayed are almost limitless and (b) unlike those using allozymes, DNA researchers use a plethora of different analytical techniques and methods of detection. Among the several advantages of DNA-level markers over protein markers include requirement of only a small amount of tissue, which could be ethanol-preserved or frozen for DNA extraction (DNA can be extracted even from formalin-preserved tissues) and the availability of innumerable potential markers. For protein markers, more amount of tissue is required, non-invasive sampling is not possible and tissue should be fresh or well frozen.

DNA polymorphisms

DNA markers are based on polymorphism detected at the DNA level. Polymorph DNA markers serve as landmarks or anchor loci for identification and analysis of new loci/genes in the genome. Polymorphism information content (PIC) is the single most important characteristic of a marker and is calculated from the allelic frequencies in the population. A PIC value greater than 0.5 is considered as highly informative, a PIC value between 0.25 and 0.5 indicates a reasonably informative marker while markers with PIC smaller than 0.25 are only slightly informative.

Categories of DNA-level markers

A discussion on the categories of DNA, based on function, structure, location etc. is given elsewhere (Jayasankar, 1997) and will not be included in the present paper. Based on their applications, DNA-level markers can be broadly put into two categories, type I and type II. Type I markers are the coding gene loci conserved across the species and are normally monomorphic or slightly polymorphic, often with two alleles. Restriction Fragment Length Polymorphism (RFLP) is an example of type I marker and contribute considerably for mapping of genes. Type II markers are highly polymorphic (PIC>0.6) and useful for population genetics and molecular taxonomy analyses. Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphisms (AFLP) and Microsatellites are examples of type II markers.

Restriction Fragment Length Polymorphisms (RFLP): Restriction enzymes are endonucleases, which occur in a variety of prokaryotes and their natural function is to destroy foreign DNA molecules by recognizing and cutting specific DNA sequence motifs typically consisting of four to six bases. Each enzyme has a particular recognition sequence, and the host bacteria usually protect their own DNA from being cut, by methylating this sequence.

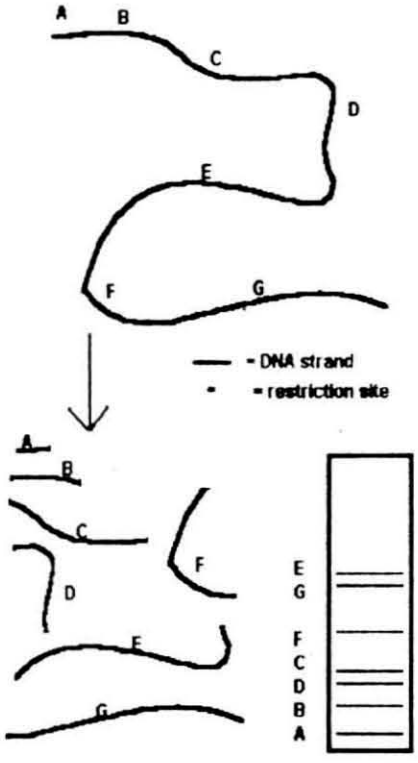

Fig. 1 Diagramatic representation of RFLP

Analysis of RFLPs for evaluating DNA sequence variation is widely used, including fisheries field. Briefly, genomic DNA is extracted, digested with restriction enzymes (a large number of them are now available commercially) and separated by electrophoresis on a gel. The gel is blotted to a nylon membrane and hybridized by a labeled probe, which is a piece of DNA. RFLP probes are locus-specific and easy to screen co-dominant markers, hence widely used for genome mapping. They can be generated from either genomic library or cDNA library.

There are two approaches to study RFLPs in the cytoplasm. The first is to extract mitochondrial DNA (mtDNA) separately from nuclear/genomic (nDNA) and digest them with restriction endonucleases, resolve it in gel and stain. The second strategy is to isolate and digest the total DNA of the organism, followed by electrophoresis and southern blotting. Polymorphisms can be visualized using specific mtDNA probes.

The advantages of RFLPs are:

- Highly polymorphic - many alleles may be present in a population for a single locus (This is an important concept - polymorphism refers to the degree of variation in the

population under consideration. Any individual can have, of course, a maximum of 2 alleles)

- Co-dominant inheritance
- Many loci can be established

The disadvantages of RFLPs are:

- The technique is laborious
- Time-consuming
- Expensive
- Usually uses isotope

Random Amplified Polymorphic DNA (RAPD): Random-amplified polymorphic DNA (RAPDs) involves the use of a single 'arbitrary' primer (purchasable from commercial companies) in a PCR reaction and result in the amplification of several discrete DNA products (Williams *et al.*, 1990; Welsh and McClleland, 1990). Each product is derived from a region of the genome that contains two short segments in inverted orientation, on opposite strands those are complementary to the primer and sufficiently close together (within 2.5-3.0 kb) for the amplification to work. In RAPDs, the amplification products are separated on agarose gels in the presence of ethidium bromide and visualized under ultraviolet light. It is now widely recognized that to obtain reproducible band profiles on the gels it is absolutely essential to maintain consistent reaction conditions.

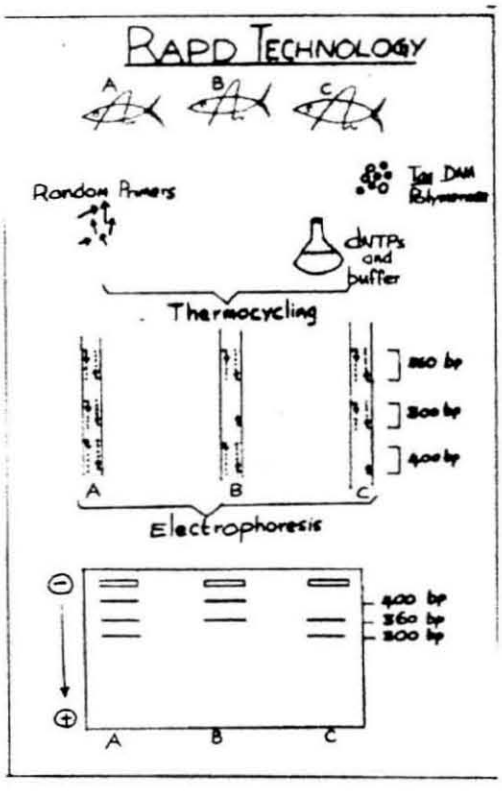

Fig. 2 Basic steps involved in RAPD

Numerous studies have reported the separate effects of altering different parameters, ratio of template DNA primers, concentration of *Taq* DNA polymerase and Mg concentration on the bands obtained (Smith, 2003). A corollary of these experiments is that RAPD profiles should be reproducible among laboratories provided that all details of the reaction conditions are standardized and strictly adhered to.

Advantages of RAPDs are:

- Rapid, simple, relatively inexpensive assay
- Many loci can be identified quickly.
- The assay can be automated.

Disadvantages of RAPDs are:

- Polymorphism is typically dominant in nature.
- Low allelic polymorphism
- Inconsistency of results

Amplified fragment length polymorphism (AFLP): AFLP is another PCR-based method which first involves restriction digestion of the genomic DNA (Vos *et al.*, 1995). Adapters are ligated

to the ends of the restricted fragments and either a pre-selection step performed using magnetic beads followed by a round of selective PCR, or two selective rounds of PCR amplification are applied. The number and composition of the selective nucleotides used as well as the complexity of the genomic DNA determine the number of resulting amplified fragments.

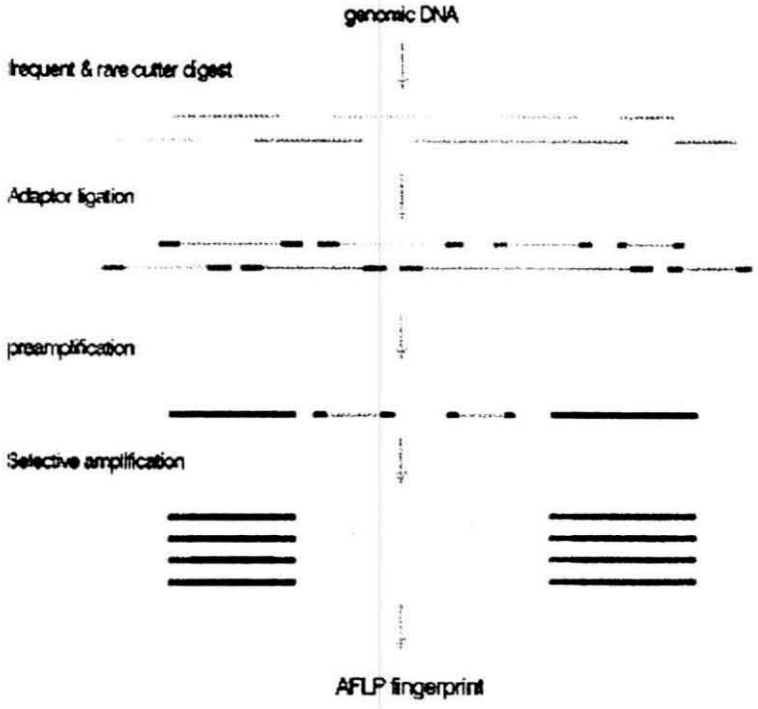

Fig. 3 Processes involved in AFLP fingerprinting

The amplified products are separated on a sequencing gel and can be visualized using radioactive or fluorescent labelling. All the current evidence suggests that AFLPs are as reproducible as restriction fragment length polymorphism (RFLP). They should therefore be highly suited to network experiments.

The advantages of AFLPs are:

- Many more bands, and so potentially many more polymorphisms, are identified than with RFLPs or even RAPDs.
- As with RAPDS, only a small amount of template DNA is needed and no probe hybridization is needed.

- Banding patterns are more consistent than with RAPDs

The disadvantages of AFLPs are:

- The method is labor intensive and requires isotope.
- The technology is proprietary.
- Bands are still scored as present or absent (i.e. dominant or recessive)

Microsatellites: Microsatellites or simple sequence repeats (SSRs) are usually one to four nucleotides long repeat units and are highly mutable loci, which may be present at many sites in a genome, occur as often as once every 10 kbp, and hence have an overall abundance on the order of 10^4-10^5 per genome (Wright and Bentzen, 1994). Some marine fishes and invertebrates exhibit 10-60 alleles per locus with high heterozygosity rates. As the flanking sequences at each of these sites may be unique, once SSR loci are cloned and sequenced, primers can be designed to the flanking sequences. The resultant sequence tagged microsatellite usually identifies a single locus, which because of the high mutation rate of SSRs, is often multi-allelic. Alleles which differ in many base pairs of length can be resolved on agarose gels but often SSRs are visualised on sequencing gels where single repeat differences can be resolved and, thus, all possible alleles detected.

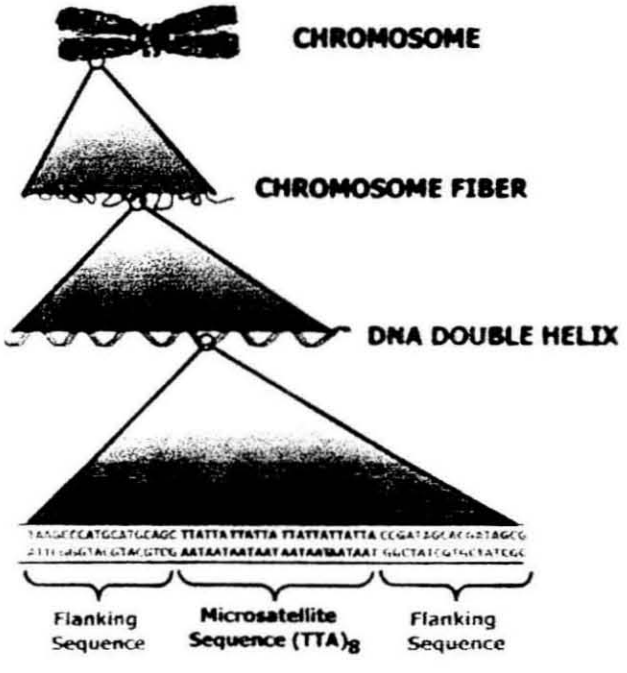

Fig. 4 Diagramatic representation of microsatellite sequences

SSRs provide highly informative markers because they are co-dominant (unlike RAPDs and AFLPs) and generally highly polymorphic. The nature of the PCR-based assay used in their amplification and detection (i.e. the use of specifically designed primers based upon the flanking sequences) suggests that they should be highly reproducible between laboratories.

Advantages of SSRs are:

- Microsatellites are easy to detect via PCR
- They generally display a great deal of polymorphism.
- They are co-dominant in nature

The disadvantages of SSRs are:

- Initial identification requires laborious screening of libraries or some other method of obtaining sequence information so that primers can be designed
- Often stutter bands appear

Mitochondrial genome: Mitochondria are cytoplasmic organelles responsible for respiratory function in eukaryotic cells. The mitochondrial genome is circular double stranded DNA with a size of 16-20 kb and containing about 35 genes. Unlike nuclear genome, mtDNA is haploid, with maternal inheritance. Several regions, particularly control region and cytochrome B gene have high evolution rate and can be used as markers for population and evolutionary genetic studies.

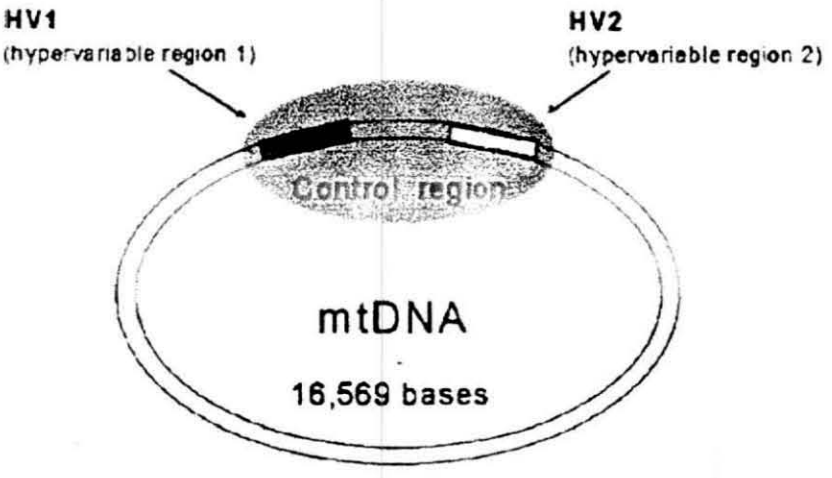

Fig. 5 Hypervariable regions of mitochondrial genome

Remarks

The recent innovations in molecular technology have increased the potential for molecular markers to provide useful information in fisheries management and aquaculture. Markers such as microsatellites have provided increased resolution power to answer stock questions in species having relatively low genetic variation. Other advances include determination of family structure, location of useful genes, etc. The specific requirement and available resources should decide choice of suitable genetic marker.

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MITOCHONDRIAL DNA AND FISH PHYLOGENY

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Introduction

Mitochondria are one of the organelles found in Eukaryotes playing a significant biological role. It originated from free living purple bacteria that became part of the Eukaryotic organism through an endosymbiotic event. The mitochondria of fishes are the least studied. The complete mitochondria genome has been sequenced and the gene order has been determined in several invertebrates and vertebrates such as drosophila, seaurchin, human, cow, frog, mouse, rat, whale, chicken, quail etc. A complete mitochondria genome has been sequenced for fish such as Atlantic cod.

The mitochondrial genome

The mitochondrial genome is a single, small double stranded circular DNA molecule present in multiple copies in mitochondria. Depending on the cell type several thousands of mitochondrial genome is found per cell. Typically the mitochondria genome is around 16500±50 bp. The vertebrates generally show a smaller size range. Interestingly, intraspecific variation is found; this is due to tandem duplication. In fishes intraspecific variation in size can be as large as differences between species.

Genetic system

Mitochondrial DNA is a unique genetic system, mt DNA is haploid and non-recombining, but in plant it appears to be mosaic i.e. a combination of chloroplast, mitochondrial and nuclear genes and is known to be recombining. The animal mt genome is exclusively maternally inherited. Paternal mitochondria appear to be actively degraded after fertilization or out replicated.

Generally one type of mt DNA is found in an organism. However, there are reports that more than one type of mt DNA is found in an individual. Heteroplasmy has been occasionally found in most major group of organisms including several groups of fishes. The heteroplasmy is due to tandem duplication of the genome, especially the D loop region, length mutation is not the only form of heteroplasmy, even single substitution is found as well. The widespread occurrence of heteroplasmy due to a size variation suggests that it might arise easily. The smaller mt DNA genome may be likely to be passed on to the next generation or out replicate the larger genome after fertilization. Though the maternal inheritance of mt genome is found in general, there are some paternal inheritance is also seen, e.g., mussel. Paternal leakage may occur at very low level, one per 1000 molecules. The molecular mechanism for this leakage is not fully understood.

Mitochondria genes

The mitochondria genome of animals contain 13 genes coding for proteins, two genes coding for ribosomal RNAs (12S and 16S rRNA), 22 genes coding for tRNAs and one major non coding AT rich region which contain sequences for initiation sites for mt replication and RNA transcription.

Mitochondria protein coding genes code for enzymes that are involved in electron transport system. They include seven subunits of NADH dehydrogenase, cytochrome b, cytochrome c oxidase, and ATP synthetase. Obviously many more gene products are required for the [illegible] mitochondria from the cytosol.

The two strands are designated as light and heavy chain. This nomenclature reflects the marked differences in the G+T content and the behaviour in cscl density gradients. With few exceptions all the genes are coded by the H strand, only ND6 and 8 tRNAs coded by the L strand.

Gene order

The mt gene order of the animals is different in each phylum; even within the phyla the differences in gene order exist. However,the piscine mt gene is not drastically different from the vertebrate consensus gene order.

Genetic code

Though the genetic code is universal, it is not the case with mitochondria DNA code, there is a slight variation. The most interesting one is the TGA codon, which acts as stop codon in nuclear genes, but it codes for tryptophan in mitochondria. The mitochondria code requires 22 tRNAs rather than 24 tRNAs of the nucleus. The piscine mitochondria code is similar to the vertebrate code.

Genetic compactness

The mt. genome is compact and efficient; there are no duplicate or non-coding sequences. Further there are no introns. The intergenic regions are not very long- but about 10 bp long in many cases. Some times the gene overlap to conserve the bases. Even the reading frames differ by 1 bp. This compactness of genes helps in the stability of the gene order. Since there are no introns and no intergenic spaces in the mt genome, there is no rearrangement of the genome. In fishes several non-coding regions have been identified, however they are very small in size.

Replication and translation

The genome replication of both the strands is initiated at two points and proceeds unidirectional without interception. The synthesis of H strand is initiated first and extends towards cytochrome b gene. Once the replicated H strand has reached the light strand origin of replication the replication of L strand begins and proceeds in the opposite direction and uses the new H strand as the template.

Replication of mt genome is continuous, asymmetric and unidirectional and therefore it requires less replication machinery. There is a single DNA polymerase gamma- this is in contrast to the typical three polymerases found in Eukaryotes. Proof reading and repair enzymes are normally absent.

Similarly transcription is also found in mitochondria. Transcription of H strand is initiated at the promoter region. The product is a poly cistronic RNA that is processed into tRNA, rRNA and mRNAs.

Evolution of mt genome

The mt genome also evolves but the rate is 5-10 times faster compared to the nuclear genes. This is confirmed by sequencing. Further faster rate of evolution is due to higher frequency of point mutation and length mutation. Originally it was thought that mt DNA is highly conserved but the sequencing result showed that it is fast changing and evolving. Many reasons are proposed for this, one is the lack of proof reading ability of the DNA polymerase gamma-lack of repair enzyme also a reason for this. There is also no mechanism to remove pyrimidines. Further greater exposure of mt DNA to oxidizing agents, supéroxide might have caused higher mutations rates. Mitochondria lack excision and recombination repair capabilities, which also contributes higher evolutionary rates. But recent studies have shown that DNA polymerase has proof reading and repair abilities. In this context whether the evolutionary rate can be attributed to lack of proof reading ability?

Substitutions

Of the mt DNA sequence changes, the substitutions play a major role. Addition and deletion are found in the control regions and intergenic spacer regions. They occur in tRNA and rRNA as well but at lower frequency. In protein coding regions the changes are rare. Transitions also play a major role. Transversions are less compared to transitions. As sequencing techniques were developed the basis for higher rate of mt DNA evolution is mainly due to transitional changes. Transitions outnumber transversions.

Transfer RNA genes

All vertebrate mitochondria genome contain 22 tRNAs. They are smaller than the cytoplamic counterparts, usually 59-75bp in length and in all cases3' CCA is not encoded, but added post transcriptionally. Still it folds into the cloverleaf structure. However, they are

variable in nature compared to their nuclear counterparts. The Evolutionary rates are hundred times faster.

Ribosomal RNA genes

Two ribosomal RNA genes are found in mitochondria genome. A small 12S rRNA (820-975bp) and a large 16S rRNA gene (1570-1640bp). Mitochondria rRNA genes evolve more rapidly than their nuclear counterparts (100 times faster). The substitutions play a major role here too. Transitions are more frequent than transversions, this is more apparent in the more closely related species. Insertions and deletions are rare but in the range of 1-5 bp.16S rRNA has more length variations than the 12S rRNA gene. Hence DNA sequences of 16S rRNA are difficult to align than the 12S rRNA genes. Length mutations are more common in this RNA than in coding regions. Fishes shows slower rate of evolutionary trends in mitochondria DNA. Cold-blooded animals show five times slower rate than the warm-blooded animals.

Mitochondria DNA and polymerase chain reaction

After the advent of PCR the knowledge about mt DNA has increased. The development of universal primers based on the conserved region has helped to study the phylogenetic relationships. With these primers mt DNA from fishes can be amplified and sequenced. The RFLP also provides enough data for population level work. RFLPs of the amplified regions can also help in population analysis.

Mt DNA and population level analysis

The fast rate of evolution of mt DNA compared to nuclear DNA makes mt DNA useful for higher resolution analysis of the evolutionary trends. This fast rate of evolution coupled with maternal inheritance has made mtDNA a popular genetic system to study gene flow, hybrid zones population structure. It is the system used for female mediated gene flow, differences in dispersal between sexes in geographical distributions. Mostly RFLP has been used, however sequence analysis gives the most reliable results. RFLP has its limitations because of the enzyme choice etc.

Phylogenetic approach

Mt DNA has been used a tool for phylogenetic studies. Mostly RFLP have been done with the whole mt genome or with the amplified products. Universal primers for PCR have generated lot of informations. Cytochrome b gene is the one used for the study greatly. The sequence homology can be used to build the phylogenetic relationship among the related species. However to get a meaningful population studies the mt DNA information with other genes has to be integrated.

RATIFICATION OF TAXONOMIC STATUS IN FISH AND SHELLFISH USING MOLECULAR GENETIC TOOLS

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Introduction

Taxonomy, the classification of living things, has its origins in ancient Greece and in its modern form dates back nearly 250 years, to when Linnaeus introduced the binomial classification still used today. Earlier taxonomists began to describe more and more species, often oblivious of each other's work, creating chaos and confusion in the whole scenario of taxonomy. This may be referred to as the first bioinformatics crisis! (Godfray, 2002). Later nineteenth century taxonomists have brilliantly solved this crisis by inventing a complex set of rules that determine how a species should be named and associated with a type specimen; how generic and higher taxonomic categories should be handled; and how conflicts over the application of names should be resolved. Current codes of zoological and biological nomenclature have evolved as a result of these efforts. Type specimens are deposited in major museum collections and are available for study. This system assures reliable and steadily updated taxonomy.

The crisis in taxonomy

However, present day taxonomy faces another series of crisis, which can be summarized below:

- Lack of adequate funding and clearly achievable goals that are both realistic and relevant
- The present day taxonomists are still deconstructing the often inadequate published works of 19th century taxonomists and scouring the world's museums for frequently poorly preserved type material
- Poor descriptions, in isolation, with the result that some of the "new" species would have been described before – with no mechanism of verification
- Heavy dependence on specialists, whose knowledge is usually lost after they retire!
- Frequent inaccessibility to specialized literature on taxonomy

In the United Kingdom, a parliamentary select committee is currently conducting an enquiry into the health of the subject for the second time in 10 years, and similar concerns are being expressed around the world (Godfray, 2002). Taxonomy can prosper again, but only if it reinvents itself as a twenty-first-century information science. It needs to adopt some of the solutions that molecular biologists have developed to cope with the second bioinformatics crisis: the huge explosion of sequence, genomic, proteomic and other molecular data.

Biotechnological solutions

Molecular taxonomy is the identification of specimens based on molecular rather than morphological characters. Molecular techniques have become a major tool for systematic ichthyologists at the species level and above. These approaches may also be useful to fishery biologists for taxonomic problems ranging between the species and population levels. For example, analysis of distribution of eggs and larvae for life history and recruitment studies of the lutjanid fishery has been hampered by the inability to identify these stages (Chow et al. 1993). Molecular genetic markers significantly increased the number of eggs and larvae that could be unambiguously identified.

The US Endangered Species Act 1973 affords protection to three categories of endangered taxa – species, subspecies and populations – but existing notions about these taxonomic distinctions based on morphological analyses have often been revised following molecular analyses (O'Brien and Mayr, 1991). Major applications of molecular markers in taxonomy include:

- Taxonomic recognition of groups showing little evolutionary differentiation Cryptic members of species complexes that can usually only be discriminated by expert morphological analysis
- Members of closely related species that can only be identified at a particular life stage
- Correct identification of the brood stock, fry, fingerlings or juveniles of cultured species
- Unambiguous identification of inter-species hybrids
- Verification of illegal fishing marketing of endangered marine mammals and elasmobranchs

In species where morphological characters overlap identification becomes difficult and tedious. The task of species identification becomes more difficult when interspecies hybridization and progressive genetic differentiation between the farmed and wild stocks due to artificial selection or inbreeding comes into play. The correct identification of the species and the confirmation of genetic purity of the populations are imperative for genetic management practices.

DNA sequence analysis is a powerful tool for identifying the source of samples thought to be derived from threatened or endangered species. Analysis of mitochondrial DNA (mtDNA) from retail whale meat markets of some Asian countries, especially Japan and Korea has shown consistently that the expected baleen whale in these markets, the minke whale, makes up only about half the products analyzed. The other products are either unregulated small toothed whales like dolphins or are protected baleen whales such as humpback, Bryde's, fin, or blue whales (Palumbi and Cipriano, 1998). Molecular based taxonomic identification has great application in validation of marketing in other species, such as sea turtles and sturgeons (Dixon *et al* 2000).

Concept of DNA-based taxonomy

DNA taxonomy system provides a new scaffold for the accumulated taxonomic knowledge and as a convenient tool for species identification and description. Figure 1 shows the steps involved in DNA-based taxonomy, which has its foundation in the collective effort of a traditional taxonomist and molecular biologist.

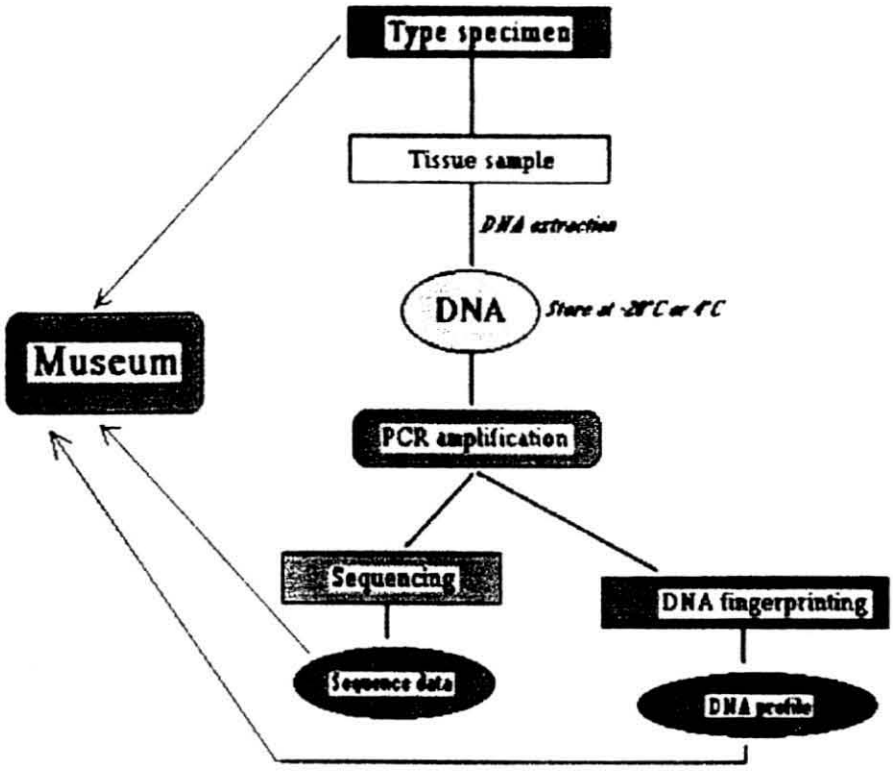

Fig. 1 Basic concept of molecular taxonomy

DNA-based system is not meant to be a critique of morphology-based taxonomy, but must be firmly anchored within the knowledge, concepts, techniques and infrastructure of traditional taxonomy (Tautz *et al.* 2003). The sequence information or the DNA profile data would serve as a standard for future reference. The type specimen, it photo and respective DNA preparation would be deposited in the museum collections. For larger specimens, a small portion can be taken for DNA extraction. However, for very small specimens, e.g. eggs or larvae, the entire tissue has to be used. In such cases, close up photograph of them should be taken. Once a significant sequence/DNA fingerprint database has been built up, new samples can be checked against these existing sequences/fingerprints to assist species re-identification or to assess whether a new species description might be warranted. The data base could also serve to resolve questions about the taxonomic identity of specimens that are derived from larval life stages, or for identification of artifacts from trade with endangered species and so on.

The binomial (comprising of one genus and species name) Linnaean naming system has an inherent instability. For example, if the genus name of a species is replaced by another name in the light of discovery of a new species or new data are evaluated which justify its inclusion into the new genus name, the older name can just disappear and only specialists might eventually be able to identify its fate. But DNA sequences/fingerprints of species can ameliorate this problem because they always provide a link to the previously used name (Tautz *et al* 2003). In case tissue samples cannot be obtained from the original specimen, newly collected individuals can be used, which are assessed by experienced taxonomists to determine their identity. It is important to follow the policy as suggested in the International Code of Zoological Nomenclature.

DNA techniques used for taxonomic purposes

Direct sequencing: Nucleotide sequence of any particular part of the genome can be obtained through high thorough-put fluorescence-based automated sequencers. Some regions are more useful than others for this purpose. The genes with the broadest taxonomic coverage currently available are those encoding the ribosomal small subunit sequences, both of nuclear and mitochondrial origin. Since this is a rather conservative gene, not particularly useful to distinguish closely related species, more quickly diverging regions, such as control region of mtDNA are more preferred. Mitochondrial *cytochrome b* gene sequences have been extensively used, particularly in apes and other mammals (Castresana, 2001). In fish and shellfish, this is not explored. Large sub unit rRNA is also faster evolving, retrievable from small and partially degraded tissues and being abundant in cells that they could serve directly as probes for DNA-microarray approaches for species identification (Pozhitkov and Tautz, 2002). In puffer fishes, 16S rRNA was used for ratification of species status (Song et al. 2001) and they found that two sibling species belonged to the same species.

PCR-RFLP: PCR can be used to amplify a homologous section of the cytochrome b region of mtDNA of different (putative) species. The resulting amplicons are subjected to restriction fragment length polymorphism analysis (RFLP), producing species-specific restriction patterns. The PCR products may also be sequenced simultaneously to substantiate correct amplification, restriction site locations and fragments sizes. Sequence data can be used to generate phylogenetic tree. Newly hatched larvae of five sympatric species of Hawaiian gobioids were correctly identified and larval morphotypes could be faultlessly assigned to the adults using this technique (Lindstrom, 1999).

RAPD: RAPDs are detected by doing a PCR (polymerase chain reaction) assay with a single short oligonuceotide primer, usually a decamer, of arbitrary sequence. Detailed account on relative merits and demerits and applications of RAPD for molecular taxonomy (Black, 1996) and in fisheries research (Smith, 2003) is available elsewhere. Since RAPD includes rapid, simple, relatively inexpensive assay, with any loci can be identified quickly and the assay can be automated, this has been the most popular method for ratifying taxonomic status of fish and shellfishes (Table 1). In the Central Marine Fisheries Research Institute, RAPD markers were effectively used to resolve taxonomic status of groupers (Govindaraju and Jayasankar, 2003) and domesticated clown fish (Jayasankar and Mathew, MS)

Unitary taxonomy

The concept of unitary taxonomy was recently proposed by Godfray (2002) and is illustrated in figure 2. The only logical way to organize a unitary taxonomy is to make it widely available on web. DNA sequence/fingerprint information is digital and not affected by subjective assessments, and can be brought onto every desktop at the click of a mouse (Bisby *et al.* 2002). Any revision proposed would be mounted on the web for refereeing and comment.

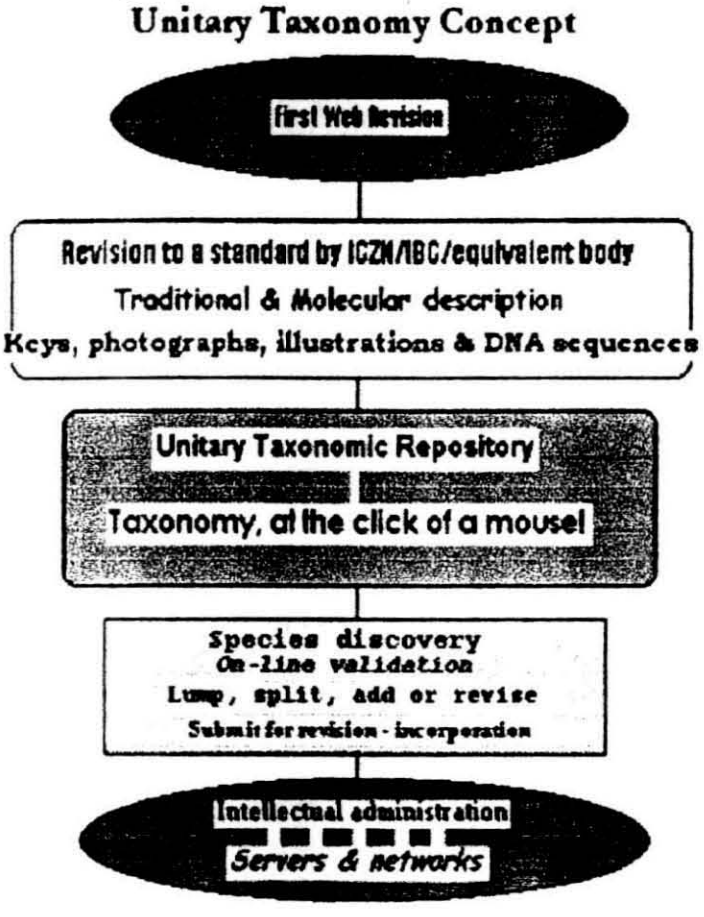

Fig. 2 Flow chart of unitary taxonomy concept

The following websites provide excellent overview of the many ongoing initiatives to capture the current taxonomic knowledge and to enhance training and expertise in taxonomy:

Box 1. Web based taxonomic initiatives

Partnership for Enhancing Expertise in Taxonomy (PEET): http://web.nhm.ukans.edu/peet/
Integrated Taxonomic Information System (IT IS): http://www.itis.usda.gov/
Species 2000: http://www.usa.sp2000.org/
Convention on Biological Diversity: http://www.biodiv.org/
Bionet International: http://bionet-intl.org/
The Tree of Life Web Project: http://tolweb.org.tree/
All Species Foundation: http://all-species.org/

Global Biodiversity Information Facility: http://www.gbif.org/
Codes of Nomenclature: http://www.biosis.org.uk/zrdocs/codes/codes.htm

Universal registration system for DNA taxonomic entries, akin to the accession numbers used in NCBI and EBI, can identify the deposited DNA sample/specimen in the museum to which a particular sequence/fingerprint relates. Individual sequences from these samples could still be submitted to NCBI or EBI, but should then clearly relate to the DNA taxonomy registration number (Tautz *et al.* 2003).

Limits of DNA taxonomy

It must be emphasized that the power of DNA sequences for identifying species is limited when species have very recent origins. Recently evolved sister species may share the alleles of the same gene, and in those cases sequences/fingerprints from one or few individuals will not be sufficient for an unequivocal assignment to a particular group (Tautz *et al.* 2003). Based on a calculation in the mutation and fixation rates in the evolution of mtDNA and nDNA, it has been estimated that the differences due to gene flow are likely to happen in species, which have evolved before about 100,000 years from now – it is safe to assume that most of the current species are older than this. There are other cases, such as cichlid fishes from Lake Victoria, where the morphological distinctiveness has built up much faster than has the molecular one; morphology-based taxonomy is clearly more powerful in such cases. Still, DNA analysis is not useless, because it provides essential insights into the time frame of the radiation and the origin of the colonizing animals (Meyer *et al.* 1990).

Conclusions

A strong taxonomic basis is indispensable for conservation of species biodiversity. Conventional taxonomic approach has been facing some serious crisis. However, recent developments and achievements in molecular biology can offer solutions to the current taxonomic crisis and would give a new impetus to biodiversity research, complementing many other ongoing efforts. One of the significant aspects of DNA taxonomy is its accessibility to millions through web and expertise of taxonomists can be easily made available. Natural history museums shall acquire modern molecular biology facilities, including high throughput sequencing equipment and thus, molecular and morphological knowledge can be formally and fruitfully combined. In the fisheries scenario, RAPD has been widely used for ratifying species status, while sequencing of specific DNA fragments has been rarely attempted. PCR amplification and sequencing of mtDNA is a powerful approach to generate molecular taxonomic data. Unless fish and shellfish taxonomy is unitary, web-based and able to accommodate the radical developments in molecular biology, it could be sidelined.

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PROTEIN ELECTROPHORESIS: PRINCIPLES AND TYPES

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Introduction

Each fish species is chemically composed of different proteins at varying levels, so techniques that separate proteins may help to identify different species. Of these techniques, electrophoresis is the most important one. Many biological molecules such as proteins are made up of amino acids with electrically charged side chains. Basic amino acids such as arginine, histidine and lysine are positively charged while the acidic amino acids such as aspartic acid and glutamic acid carry negative charges. Thus, virtually all proteins have a net charge depending on the relative proportions of amino acids, unless they are at their "iso-electric point" (pI), the definite pH at which the net charge of the protein molecule is zero. The basis of electrophoretic separation is that proteins of different net charge and different molecular size will migrate at different rates within an electric field and it is a very useful technique for the separation of cellular proteins and DNA.

The term electrophoresis comes from the Greek, and means, " transport by electricity" and has been known since the end of 19th century. In 1807, a Russian Physicist, Alexander Reuss observed a novel phenomenon - when electricity was passed through a glass tube containing water and clay, colloidal particles moved towards the positive electrode. The term electrophoresis describes the migration of a charged particle under the influence of an electric field. Charged molecules are having either positive or negative charge. At a given pH, the biological molecules exist in solution as electrically charged particles. Under the influence of an electric field, these charged particles will migrate either to the cathode or to the anode, depending on the nature of their net charge.

The theory of movement of a particle in electrophoresis is as follows: When a potential difference (voltage) is applied across the electrodes, it generates a potential gradient, **E**, which is the applied voltage (**V**) divided by the distance, **d**, between the electrodes. The force that drives a charged molecule towards an electrode is the product of potential gradient, and the charge of **q** coulombs on the particle. However, the frictional force that retards the movement of a charged molecule, is function of hydro-dynamic size of the molecule, shape of the molecule, the pore size of the medium in which electrophoresis is taking place and the viscosity of the buffer.

The velocity (**v**) of charged molecule in an electric field-

$$v = \frac{Eq}{F}$$

where **F** = frictional coefficient, which depends upon the mass and shape of the molecule.

E = electric field (V/ cm)

Most of the large molecules possess both anionic (basic positively charged) and cationic (acidic-negatively charged) groupings as part of their structure and hence are termed as "amphoteric molecules" or "Zwitterions". The actual charge of protein molecule is the result of the sum of all single charges. Because dissociation of the different acidic and basic groups takes place at different hydrogen ion concentrations of the medium, pH greatly influences the total charge of the molecule. At lower pH, they migrate to the negative pole (cathode) and at higher pH to the positive pole (anode). Ionic strength also affects the migration, low ionic strength permits high rate of migration. The choice of buffer strength may be seen to be crucial, since it determines the amount of electrical power that can be applied to the system. The rate of migration will also depend upon the charge density (the ratio of charge to mass) of the proteins concerned; the higher the ratio of charge to mass, the faster the molecule will migrate.

Gel electrophoresis

In this type of electrophoresis, gel is used as the support media instead of thin paper sheets etc. Most electrophoresis is carried out on the inert media.

Types of gel electrophoresis

I. Based on Buffer System

II. Based on Support media

I. Based on Buffer System

A. Continuous buffer System:

Continuous buffer electrophoresis is the simplest and most commonly used method. The same buffer is used to provide electrical contact between the platinum electrodes and the support medium and to soak the gel. In the gradient pore method an acrylamide gel is prepared other. Protein solutions are applied at the end of the gel where pore size is largest and allowed to migrate in an electrical field until they reach the point where pore size prevents further movement. Separation is purely on the basis of molecular size and electrical charge is used only to induce movement.

B. Discontinuous buffer System:

In discontinuous buffer or multiphasic electrophoresis, the electrode chambers contain a different buffer to that in the gel. The front at which the two buffers meet concentrates different proteins so that they enter the main electrophoresis system as a very narrow zone. This will enhance the resolution.

II. Based on Support media

Supporting media: A variety of anti-convection media are being used in zone electrophoresis. They exhibit several properties. An ideal medium should have the following features: -

Chemical nature	inert
Availability	easy
Electrical conductivity	high
Adsorptivity	low
Sieving effect	desirable

Thus, electrophoresis, a ubiquitous biochemical method that allows separation as well as visualization of macromolecules. This may be considered as the core technique of all molecular based studies. In the filed of genetics, it may be utilized as the base technique in examining the genetic diversity of individuals/population, which in turn may help in establishing genetic relatedness between taxa to provide major role in conservation and management strategies.

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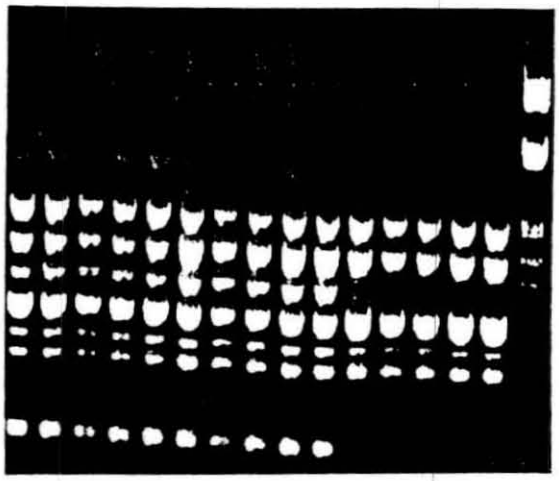
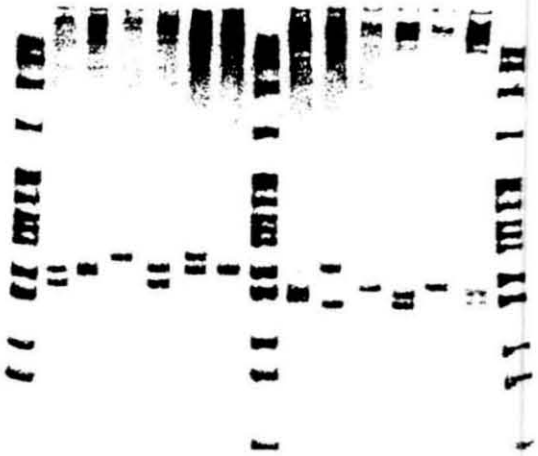
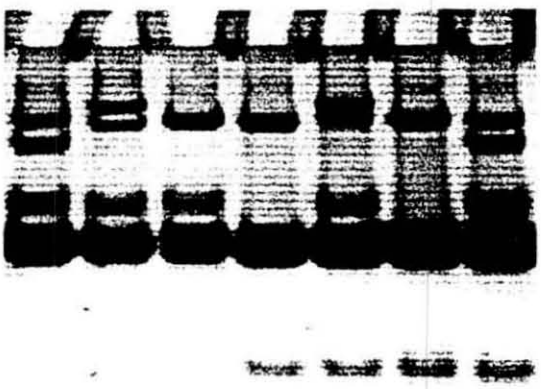
Fig. 3. Images of different types of gel electrophoresis.



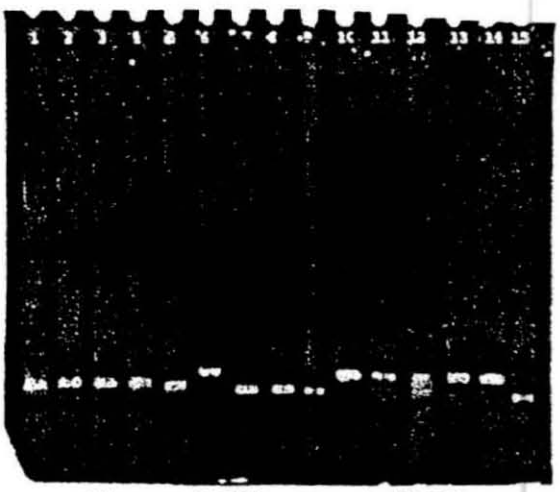
RAPD pattern of fish DNA with Operon primer
Agarose (1.5%) electrophoresis



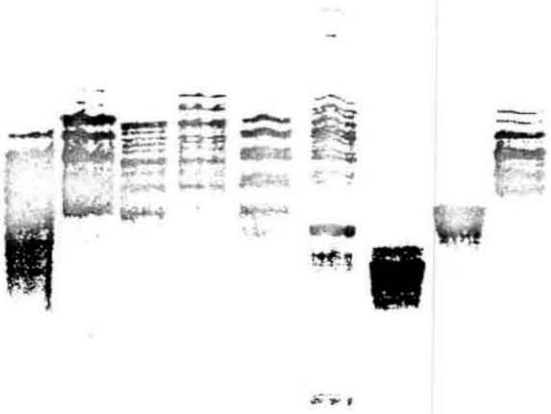
Microsatellite pattern of fish DNA in PAGE
with silver staining.



Allozyme (Esterase) pattern in PAGE



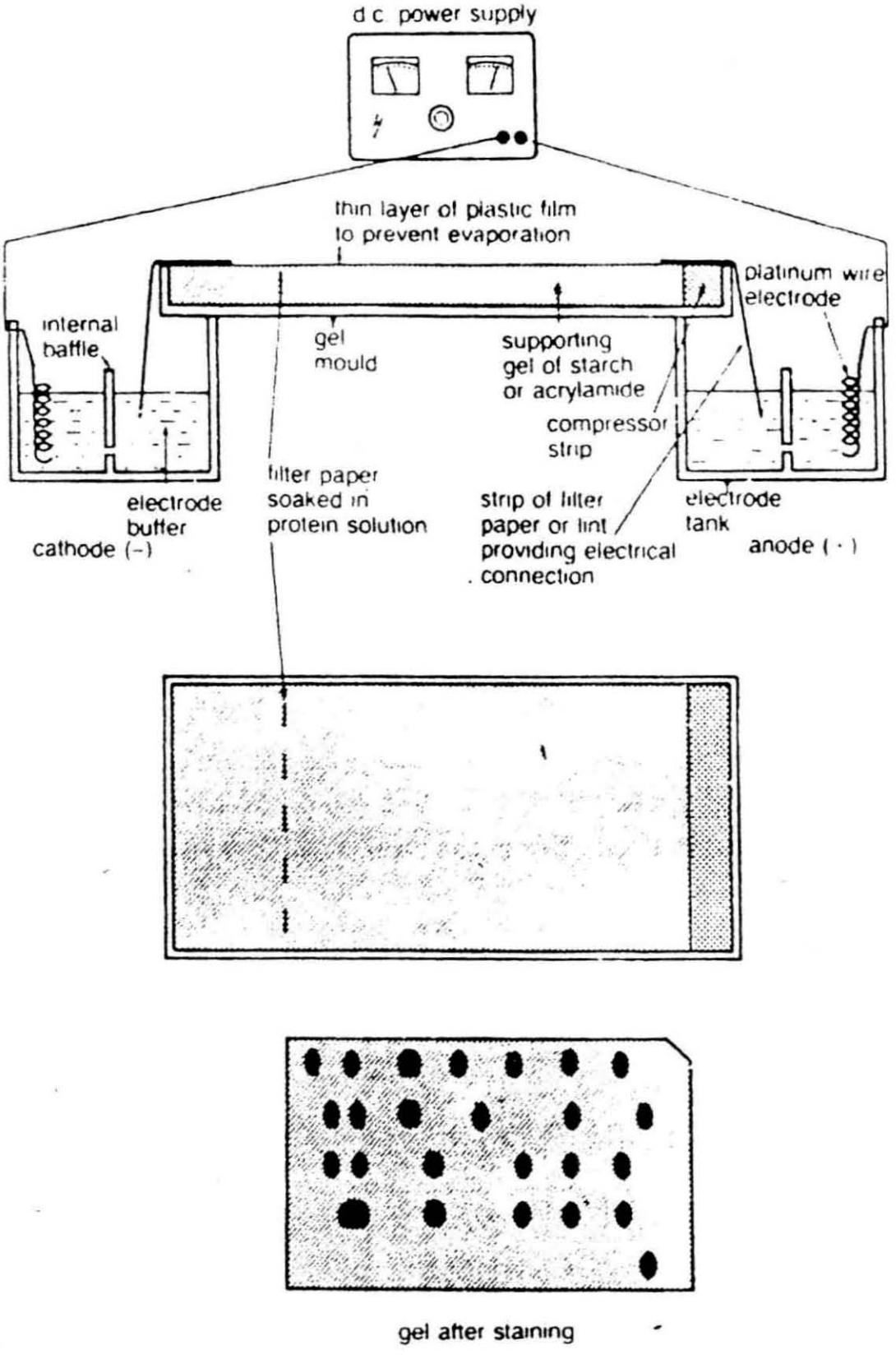
Allozyme (SOD) pattern in PAGE



Ultra-thin IEF of fish haemoglobin

2D gel electrophoresis of frog oocytes
(IEF and SDS PAGE at right angles)

Fig. 2. Horizontal Gel Electrophoresis



gel after staining

necessary reagents, to the staining mixture (figure 1C). The most frequently used technique is to identify reactions involving hydrogen ion transfers from the substrate to nicotinamide adenine dinucleotide- (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) (i.e. to convert NAD to NADH or NADP to NADPH). These are called dehydrogenase reactions. Phenazine methosulphate (PMS) is then used to transfer the hydrogen ion from the NAD to one of the tetrazolium salts, usually methyl-thiazolyl blue (MTT) or nitro blue tetrazolium (NBT). The soluble, weakly yellow coloured, tetrazolium salts are reduced to insoluble blue formazan, which appears as a purplish zone on the gel wherever the reaction occurs.

Specific enzymes can be stained in this fashion. Particular dehydrogenases are detected by the addition of the correct substrates for the enzyme along with PMS and MTT (figure 1B). Enzymes that can be coupled to a dehydrogenase can also be stained by using the correct combination of substrates and linking enzymes (e.g. figure 1C).

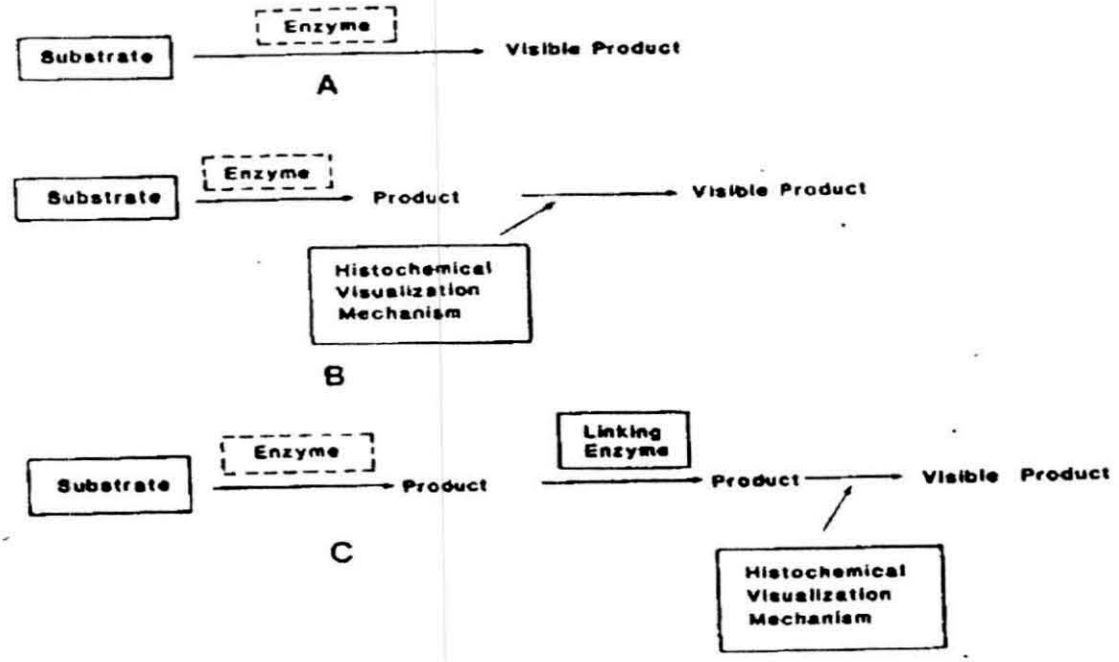


Fig.1. Types of staining systems for enzymes.

A. The enzyme being stained for converts the substrate directly into a visible product.

B. The enzyme being stained for converts the substrate into a product that is not visible, but can be made visible by the addition of other histochemicals.

C. The enzyme being stained for converts the substrate into a product that is not visible but can be converted by a linking enzyme into a second product that can be made visible.

many fish species. Allozyme electrophoresis has been used in defining genetic markers for stock identification on the basis of differences in allelic frequencies between stocks in many species. Using allozyme markers, it is possible to determine whether a population is a random mating one with equilibrium genotypes frequencies or sample comprises of an assembly of genetically distinct units. Their allele frequencies primarily respond to mutation, gene flow and drift. One of the limitations of enzyme variants as genetic markers is the low level polymorphism observed in some species and populations. The extensive allozymes studies undertaken on f1sh stocks have not only proven valuable for estimating population divergence, but also have focused attention on the underlying evolutionary forces that promote differentiation.

Staining Systems: Once a gel has been run, it is necessary to identify the positions of the protein products of a particular locus. Most proteins are colourless and way to detect the separated bands is by staining them like *histological specimens* using the histochemical stains developed by histologists. For example, to detect all forms of protein, the technique is to first denature them and 'fix' them in position in the gel by fixation with an acid. They are then stained and, finally, excess stain washed from the gel, much as a tissue specimen is processed for microscopic examination. The pattern of stained bands is called 'zymogram'. Proteins are usually stained with wool dyes, such as Ponceau red S, Amido black 10 B, Kenacid blue or Coomassie Brilliant Blue R. Other stains may also be used, as in histology, to locate a general class of proteins, *e.g.* the alcian blue stain for acid mucopolysaccharides and Oil red O for lipoproteins.

There are major shortcomings in the use of such non-specific protein stains. Normally a large number of zones are stained and, without functional information for each zone in each sample, the relationships between the zones are difficult to determine. As a consequence, the number of loci and alleles involved in the patterns observed can rarely be determined.

An alternative approach is to locate areas of the gel containing specific proteins by using specific histochemical staining techniques in which some functional property of the protein is used to detect its presence. These techniques vary widely, being limited only by the ingenuity of research workers. For example transferrin, the iron-transporting protein of vertebrate serum, may be detected by adding radioactive iron to the sample before electrophoresis, and then identifying the location of the protein-bound radioactive iron after electrophoresis.

The most common staining techniques used in electrophoresis detect particular enzymes. Since each enzyme catalyses a specific reaction, any enzyme can be histochemically localized provided that either a substrate or, more commonly, a product involved in its reaction can be made visible (figure 1A and B). Coloured bands appear on the gel wherever product is formed by the action of the relevant enzyme. When the substrate is detected, the entire gel is coloured, except for areas where the substrate has been used up by the enzyme. Enzymes can also be detected by coupling the reaction so catalysed to one that forms a product, which can be detected. This is achieved by adding an appropriate *linking enzyme,* along with other

at –40°C most tissues will remain usable for a year or more, but at –18°C denaturation is relatively rapid and will produce altered electrophoretic pattern.

Proteins studied for fish species identification: -

1.Sarcoplasmic proteins (water soluble proteins): The soluble proteins of the sarcoplasm, located within the sarcolemma are referred to as sarcoplasmic proteins. Among them, some albumins and so called myogens; to which belong most of the glycolytic enzymes are the real water-soluble proteins. (The other fractions of sarcoplasmic proteins are soluble in low salt concentrations). The genetic differences between species are more pronounced in this than in other group of proteins, as they are responsible for widely divergent enzymatic transformations in the muscle cell. Hence, the separation patterns of profiles obtained on electrophoresis or isoelectric focusing (IEF) can be used for the unequivocal identification of the species.

2. Myofibrillar proteins or salt soluble proteins: They are salt soluble proteins present in the myofibrils of the muscle fibre. Of the different myofibril proteins, myosin and tropomyosin find application in fish species identification by electrophoresis. Fish myosin, similar to myosin of other vertebrates, is a hexameric protein consisting of two identical heavy chains and four light chains, of which two of them are identical. Electrophoretic pattern of heavy chains from different species are similar whereas that of the light chains is different for different species. Hence, an electropherogram of myosin light chain isolated from fish muscle is used for species identification. Electrophoresis of most of the fish muscle tropomyosin gives a single band whose electrophoretic mobility is different for different species. Tropomyosin is a heat stable protein that can be extracted from heat-treated fish products, thus useful in identifying the species of fish of the product by studying the SDS-electrophoretic pattern of tropomyosin.

3. Eye-lens Proteins: The soluble protein of the eye-lens have great value in taxonomic studies, because they are synthesized only one cell type present in the eye as a single layer. Three saline soluble eye lens proteins are distinguishable by electrophoretic and immunological techniques. There are alpha-beta-, and gamma crystalline in order of decreasing electrophoretic mobilities, each of which constitutes a family of similar, but no identical proteins. Protein with alpha-crystallin characteristics have been found in all vertebrate species and regarded as a classical organ-specific protein. The beta- and gamma- crystallin patterns are species specific and can be used to resolve taxonomic disputes using ultra-thin IEF technique.

4. Allozymes: Isozymes are functionally similar and separable forms of enzymes encoded by one or more loci. Isozyme products of different alleles at the same locus are termed as "allozymes". The most important quality of allozyme data is the codominant nature of inheritance of gene products and thus genetic interpretation (genotype) of the phenotype is facilitated because all products are normally visible and not masked by dominance of one over another. Other advantages include function of most of the proteins are known and, extensive database is available for

electrophoresis power supply. These equipments are made to supply constant voltage, current or power.

Constant Voltage: Almost all power supplies provide constant voltage. Voltage gradient of 15/cm is generally set for electrophoresis at room temperature (25^0C). But, when higher voltage is employed, heat generation is unavoidable. Hence for all types of electrophoresis using agarose gel, which is heat labile, a constant low voltage is given. Increased resistance during the run is reflected in the decrease of mA.

Constant Current: This provision is available in imported power supplies, and is generally required for Disc-PAGE to generate localized voltage gradient. Upto 5 mA/gel rod and 25 mA/gel slab is provided. Voltage and temperature rise during electrophoresis can be lowered by buffer circulation through a coolant.

Buffers:

It is a solution of a weak acid and one of its salts. It resists changes in H^+ and OH^- ion concentrations and maintains constant pH. Each buffer has its own 'buffering capacity' (the rate of change of pH to the number of equivalents of acid or base added). The following are the commonly employed buffers and their pH values with regard to electrophoresis.

Buffer	**pH value**
Phosphate buffer	around 7.0
Tris-Borate-EDTA buffer (TBE)	around 8.0
Tris-Acetate EDTA buffer (TAE)	above 8.0
Tris Glycine buffer (TG)	more than 8.5
Tris -Citrate-EDTA buffer (TCE)	around 7.0
Tris -EDTA buffer (TE)	around 8.0
Tris -Maleic acid -EDTA buffer (TME)	around 7.5
Lithium Borate - buffer (LB)	around 8.6

Sources and extraction of proteins: -

For electrophoretic separation, proteins must be in solution. These can be body fluids such as plasma, serum, milt, haemolymph or aqueous extracts of tissue proteins. Extracts are often made from muscle, liver, eyes, etc in distilled water, 10% sucrose solution or specific extraction buffer. Crustaceans can be sampled non-lethally by removing a walking leg. With larvae or other small organisms, it is necessary to use the whole animal. The pH and ionic concentration of the buffer used, permits differential extraction of proteins.

After removal from the animal, proteins begin to denature rapidly and so the tissue must be used immediately or stored deep-frozen. Storage at –196°C in liquid nitrogen or at –80°C in ultra low freezers retains 90% of initial activity indefinitely and

to 3% is most effective for nucleic acid separations. Gels with agarose concentration less than 0.5% are rather fragile and must be used in a horizontal slab arrangement or in a refrigerated chamber. Like proteins, nucleic acids migrate at a rate that is inversely proportional to the logarithm of their molecular weights; hence molecular weight can be estimated from electrophoretic results using standard nucleic acids of known molecular weight. Passage of a molecule through a gel is influenced by the shape and size of the molecule. A small, compact molecule would be expected to have a greater mobility than rod like, linear molecules.

Most agarose gel electrophoresis experiments are carried out with horizontal slab gels. This method is chosen over vertical mode because low agarose concentration can be used for maximum mobility.

D. Cellulose acetate gel electrophoresis:

In this type of electrophoresis, protein migration essentially takes place in the buffer film on the gel surface. The medium has therefore no influence on the electrophoretic mobility.

E. Isoelectric Focusing (IEF):

Isoelectric focusing uses a polyacrylamide gel with large pore size containing a mixture of polyamino, poly carboxylic acids with different isoelectric point (pI) s. These form a stable pH gradient along the gel in an electric field. Strong acid applied at the anode and strong base in the cathode contain and stabilize the gradient. Proteins migrate under the influence of their charge until they reach the point in the gel where the pH is equivalent to their isoelectric point and so their charge is neutralized. At the isoelectric point, proteins in the electrical field do not migrate to either of the poles. High resolutions are achieved permitting separation of proteins differing only by 0.01 pI.

F. Two - dimensional (2D) electrophoresis:

The techniques of isoelectric focusing and polyacrylamide gel electrophoresis have been combined to produce two-dimensional separation of proteins. This technique is increasingly used now a days and its great resolving power is due to the use of two independent properties of proteins. The proteins are first separated by isoelectric focusing (this is the first dimension), which separates proteins according to their charge (isoelectric point). The proteins are subsequently separated by SDS-PAG electrophoresis (this is the second dimension) at right angles, which separates proteins according to their size (molecular weight). This technique results in a series of spots distributed throughout the polyacrylamide gel.

Source of current:

The source of DC is a simple battery. However, for prolonged and constant supply, alternating current (AC) after rectification to DC is employed. Unlike in DC, the electron flow in AC is not unidirectional. But this is rectified by equipment called

tetra methylenediamine (TEMED) as initiator. Out of the two, the most used is TEMED and proportional increase in its concentration speeds up the rate of gel polymerization. Gelation occurs due to vinyl polymerization. Prior degassing of solution is required since molecular oxygen inhibits chemical polymerization. The relative proportion of acrylamide monomer to cross-linking agent determines the porosity of a gel. Gels may be defined in terms of the total percentage of acrylamide present. Gels may be prepared containing from 3% to 30% acrylamide, corresponding to pore sizes of 0.5nm and 0.2nm, diameter respectively. Polyacrylamide gels may be prepared with a high degree of reproducibility and the precise porosity. This feature makes the method particularly suitable for resolving mixtures of proteins. This feature makes the method particularly suitable for resolving mixtures of proteins. Other features of polyacrylamide gels include their minimal absorption capacity, their lack of electro-endosmosis and their general suitability for *in situ* quantitative analysis (as they do not absorb UV) and for various types of histo-chemical analysis. For running of the polyacrylamide gels, the gel slab (earlier gel rods made in glass tubes of uniform diameter were used) is loaded with the sample and attached to the lower submarine unit, filled with the buffer and connected to the power pack for the DC supply.

2. **Denaturing Polyacrylamide gel electrophoresis: -** In the electrophoretic technique previously discussed, the mobility of biological molecules is influenced by both charge and size. But, if protein samples are treated with certain chemicals so that they have a uniform charge, the electrophoretic mobility then depends primarily on size. The molecular weights of proteins may be estimated if they are subjected to electrophoresis in presence of detergent, SDS and disulfide reducing agent mercaptoethanol. When protein molecules are treated with SDS, the detergent disrupts the secondary, tertiary and quaternary structure, leaving the molecule to produce polypeptide chain in a random coil, imparting an overall negative charge and masking the individual variation in charge. The presence of mercaptoethanol assists in protein denature by reducing all disulfide bonds. In essence, polypeptide chains of constant charge/mass ratio and uniform shape are produced. The electrophoretic mobility of the SDS-protein complexes will be influenced primarily by molecular size; the larger molecules will be retarded by the molecular sieving effect of the gel, while the smaller molecules will have greater mobility.
   In practice, a protein of unknown molecule weight and structure is treated with 1% SDS and 0.1 mercaptoethanol in electrophoresis buffer. A standard mixture of proteins with known molecular weights must also be subjected to electrophoresis under the same conditions. After electrophoresis followed by staining, the molecular weight may be determined.

C. Agarose electrophoresis:

The electrophoretic technique used to characterize DNA and RNA is through agarose gels. The mobility of nucleic acid in agarose gels is influenced by agarose concentration and the size and shape of the nucleic acid. Agarose concentration of 0.5

Porosity	controlled
Transparency	high
Electro-endosmosis (EEO)	low
Rigidity	moderate to high
Preservation	feasible
Toxicity	low
Preparation	easy

The original material, filter paper, has now been replaced by a variety of gels. Cellulose acetate and agar have large pore size and are used for separation of large protein molecules and for immunodiffusion. Starch and polyacrylamide have a pore size to the molecular size of many proteins, so there is also a molecular sieving effect. The pore size of acrylamide gels is adjustable. Protein resolution depends on the pH and ionic strength of the buffer, the pore size of the gel and the current applied. Buffer pH is usually 8 to 9 ensuring that all proteins are negatively charged and migrate to anode. Heat is produced in proportion to the product of Volt x Ampere so temperature must be controlled by using cooling devices. The details of various media are discussed below.

A. Starch gel electrophoresis:

In this type of electrophoresis, starch is used as the support media. The molecular sieving properties of starch make it good choice for the separation of complex mixtures of structural molecules and physiologically active proteins. Starch gel is prepared by mixing hydrolyzed potato starch with an electrolyte buffer, cooking until a gel of uniform consistency is achieved, followed by pouring the gel into a mold. The ends of the longer section of the gel are placed gently on the absorbent towels or filter paper in the lower (anodal) buffer tank. Each tank is approximately one-third filled with chilled electrode buffer.

Voltage, current and duration of the electrophoresis and other requirements vary widely among buffer systems for particular electrophoretic separations. Gels should be run in the cold chambers to produce sharp and straight lines of migration and to minimize heat production in overnight conditions usually requiring a constant 200V and variable current of not more than 30mA. An important application of Starch gel electrophoresis is the analysis of isozyme pattern.

B. Polyacrylamide gel electrophoresis (PAGE):

1. **Native polyacrylamide gel electrophoresis-** Acrylamide monomer (CH_2= CH CO NH_2) is copolymerised with a cross linking agent, usually N.N'- methylene bisacrylamide {CH_2 (NH CO CH = CH_2)$_2$}, in the presence of a catalyst accelerator chain initiator mixture. This mixture consists of freshly prepared ammonium per sulfate as catalyst (0.1 to 0.3% w/v) together with about the same concentration of a suitable base, for example, dimethyl aminopropionitrile (DMAP) or N, N, N', N' -

THE PRINCIPLES OF ISOLATION, PURIFICATION AND ANALYSIS OF NUCLEIC ACIDS

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Introduction

Advanced Biotechnological research is largely depended on the genome analysis and recombinant DNA technology. Good quality nucleic acid is an essential prerequisite for consistent results in most of the down stream applications in the genome analysis and recombinant DNA technology. The general principle underlying the isolation of nucleic acids is common with few modifications depending on the type of nucleic acids being isolated. The type of the nucleic acid intending to isolate is to be made free from the other biological macromolecules and cell debris. This is achieved by properly lysing the cell wall or cell membrane as the case may be and by selectively denaturing the other macromolecules like proteins. Nucleic acids thus recovered in its native form are to be purified by removing the very closely associated molecules. The finely purified molecule is precipitated by alcohol and suspended in sterile buffer or distilled water. Finally, the qualitative integrity of the isolated nucleic acids is to be checked by agarose gel electrophoresis and ethidium bromide staining before proceeding with the further downstream applications. Quantitative estimation of nucleic acids are carried out by spectrophotometric and fluorimetric methods.

The types of nucleic acids usually isolated on a routine basis are:

1. Total genomic DNA
2. Total RNA
3. Plasmid DNA & Mitochondrial DNA

A) Total Genomic DNA

Breaking of the bacterial and plant cell walls as well as solubilising the cell membrane of animal tissue are to be carefully carried out under optimum conditions. Even the rapid stirring of solution can break high molecular weight DNA into shorter fragments. Vigorous shaking will cause nicks and even cut open the covalently closed circular structures of plasmid and mitochondrial DNA. If physical disruption is necessary as is the case with certain types of tissues, it should be kept to the minimum, and should involve cutting or squashing of cells, rather than the use of shear forces. Ultra sonic sounds are used to disrupt the tough cell wall of certain bacteria. Care has to be taken to prevent degradation of DNA by deoxyribonucleases. These enzymes are found in most cells, and may also be present in dust, which could contaminate laboratory glasswares. Hence all the glass wares, plastic wares and the homogenizing buffer are to be made sterile by autoclaving. Using

EDTA in buffers, which will chelate the Mg++ ions needed for DNase activity, can inhibit this enzyme activity. Cell disruption and most of the subsequent steps should be performed at 4°C.The cell wall could be lysed enzymatically as well. The enzyme Lysozyme usually lyses the bacterial cell wall. The cell membranes on the other hand are solubilised by including suitable detergent in the homogenizing buffer. Upon lysis the nucleic acids will be released into the cytoplasm and now the target molecule, DNA, is to be made free from RNA and other associated proteins. The RNA molecules can be selectively denatured by enzymatic treatment with RNase. Prior to its use, the RNase is to be heat treated to inactivate any DNase contaminants. RNase is relatively stable to heat as a result of its disulphide bonds, which ensure rapid renaturation of the molecule on cooling. The other major contaminant, protein, is removed by enzymatic treatment with proteinase K followed by shaking with water-saturated phenol or with phenol-chloroform mixture, either of which will denature proteins but not nucleic acids. Centrifugation of the emulsion formed by this mixing produces a lower, organic phase, separated from the upper, aqueous phase by an interface of denatured protein. It is advisable to use cut micro tips while proceeding through these steps. The aqueous solution is recovered and deproteinised repeatedly until no material is seen at the interface. Finally the deproteinised DNA preparation is mixed with two volumes of absolute ethanol, and allowed to precipitate out of solution in a freezer. After centrifugation, the DNA pellet is redissolved in a buffer containing EDTA for protection against DNases, and this solution can be stored at 4°C for at least a month. DNA solutions can be stored frozen, but repeated freezing and thawing tends to damage long molecules by shearing and hence the DNA preparations in frequent use are normally stored at 4°C.

B) Plasmid & Mitochondrial DNA

The principle of isolation of plasmid and mitochondrial DNA is based on the structural characteristics. Plasmids are double stranded, Covalently Closed Circular (CCC) or super coiled structures. Similarly mt. DNA is also having the same structural characteristics and hence almost the same isolation procedure can be adapted. Bacterial cell wall is to be broken by enzymatic treatment (lysozyme) in a suitable buffer with a suitable metallic chelator like EDTA before initiating the isolation process. The tissue for the mtDNA isolation is to be thoroughly homogenized under ice-cold conditions.

The classical method is to isolate the plasmid and mitochondrial DNA by Caesium chloride density gradient ultra centrifugation in the presence of ethidium bromide. Ethidium bromide causes unwinding of DNA as it binds to it, simultaneously producing a decrease in its buoyant density. Since the super coiled plasmid and mtDNA can unwind to only a very limited extent, it will not bind as much ethidium bromide as with the linear and open circle forms of DNA in the presence of saturating levels of ethidium bromide. Because of this density difference, plasmid and mt DNA can be separated from other DNA by ultra centrifugation.

Another method, which is relatively fast, is based on alkaline lysis. In this method the property of super coiled DNA to remain intact at pH between 12 and 12.5 is exploited for the isolation. At this pH selective denaturation of linear DNA will occur where as the super coiled DNA will remain intact. Further reduction of the pH to acidic condition will enhance the formation of a complex network of proteins and linear DNA and the resultant supernatant after centrifugation will contain the intact plasmid or mtDNA. This can be purified and precipitated as in DNA isolation procedures. For mitochondrial DNA, this method works well with fresh tissues with minimum nicks.

Mitochondrial DNA can also be isolated by differential centrifugation technique. This involves the selective isolation of the mitochondria, which is further lysed with suitable detergents to release the mtDNA. This will be further purified and precipitated by conventional means.

C) RNA

RNA molecules are relatively short, and there fore less affected by shearing. RNA is, however, very vulnerable to digestion by RNases which are present abundantly even on fingers. These enzymes are stable and generally require no co-factors. Hence gloves should be worn, and a strong detergent should be included in the isolation medium to denature any RNases immediately. The solutions used are to be treated with nuclease inhibitors like Diethyl pyrocarbonate (DEPC). Care should be taken while using DEPC, as it is a suspected carcinogen. Glasswares should be baked at 300°C for 4 to 5 hours, as autoclaving alone may not be sufficient to fully inactivate RNases. The plastic ware can be rinsed with chloroform. Tissue homogenization is to be carried out under ice-cold conditions with all the precautions detailed above. As in the case of DNA, RNA is to be made free from DNA and proteins. Proteins are denatured by proteinase K treatment followed by phenol chloroform extraction. This is followed by the ethanol precipitation of RNA in the presence of sodium acetate or sodium chloride. The over night precipitated pellet is washed with 70% ethanol to remove the salts and finally dissolved in DEPC treated water. Contaminating DNA can be removed by treatment with RNases free DNase. The RNase can be inactivated by RNAsin or vanadylribonucleoside complex.

Commercially available kits

Several readymade kits are available commercially and many laboratories are depending on such products for the isolation of nucleic acids. In most of these kits, the nucleic acids are either trapped by ultra filtration membranes or allowed to bind with certain resins, which have affinity towards nucleic acids. The advantage with these kits is that the process is very fast and devoid of using corrosive organic chemicals like phenol. The main disadvantage is that they are quite expensive and hence unaffordable to many laboratories. Hence it is advisable to use alternative non organic protocols, for DNA isolation, based on the use of high concentration of salts for removing proteins in place of phenol, which are

easy to perform in the laboratories especially while isolating from liquid connective tissues like blood, haemolypmph etc. Meanwhile, the commercial kits are effectively used for the isolation of total RNA and mRNA, as the manual isolation is a sensitive process with increased chances of degradation.

Quantitative Estimation of nucleic acids

DNA and RNA can be spectrophotometrically estimated by taking optical density (OD) at 260nm, 1O.D corresponds to 50 micro gram of DNA and 40 micro gram of RNA. Purity of the DNA can also checked spectrophotometrically by taking O.D at 260 &280nms. The ratio of 260 and 280 will result a value of 1.8 with pure nucleic acid preparations.

Suggested readings

Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). Molecular cloning. Cold Spring Harbor ,New York

Old, R.W. and Primrose, S.B. (1985). Principles of Gene manipulation,3rd edition. Blackwell Scientific Publications,Oxford.

Walker, J. M.and Gaastra, W. (Eds) (1983).Techniques in Molecular biology. Croom Helm,London.

THE POLYMERASE CHAIN REACTION

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Introduction

The Polymerase Chain Reaction (PCR) is a technique for the *in vitro* synthesis of billions of copies of a specific nucleic acid sequence by performing successive rounds of *in vitro* nucleic acid replication. This is achieved by using two oligonucleotide primers that hybridize (annealing) to the opposite strand of the target DNA at positions that flank the region to be amplified through simultaneous extension of both primers. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesized in one cycle can serve a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR, yields about million-fold amplification.

The PCR with tremendous applications was possible because of the availability of nucleic aid sequence information and the thermostable DNA polymerase enzyme. The components required for the PCR viz., the template (the DNA to be amplified), the primers, *Taq*. polymerase, the four types of de-oxynucleotide triphosphates and buffer containing magnesium ions are assembled in a tube and the amplification reaction carried out by cycling the temperature within the reaction tube. For any given pair of oligonucleotide primers, the optimal concentrations of all the above ingredients and parameters have to be standardized. Even though, there is no single set of conditions and concentrations that will be optimal for all reactions, the parameters outlined below defines a common starting point from where modifications can be attempted.

The standard PCR mixture in addition to the sample (template) DNA contains 50mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 µg/ml gelatin, 0.25-100 p moles of each primer, 200 µm of each deoxyribonucleotide triphosphate (dATP, dCTP, dGTP and dTTP) and 2.5 units of *Taq* polymerase. The sample DNA generally contains 10^2 to 10^5 copies of template. The volume is made up to 25 or 50µl. The amplification is performed in a DNA thermal cycler, each cycle consisting of denaturation at 94°C for 30- 60 sec, annealing at 55°C for 30- 90 sec and extension at 72°C for 60- 120 sec for total of 30 cycles. Cycling could include an initial denaturation at 94°C and a final extension at 72°C for 5 min. At the end reactions are stopped by chilling at 4°C or by addition of EDTA at 10mM.

Materials and Reagents for PCR

Target DNA (Template)

An advantage of PCR is that it can amplify relatively impure DNA or DNA from blood spots, archival material and ancient DNA. The nucleotide composition of target DNA also affects the PCR amplification. Extremely GC rich DNA strands are difficult to separate. Addition of denaturing agents like formamide or DMSO can help to overcome the problem. Concentration of template DNA also affects the degree of amplification. Too high or too low concentration will result in poor amplification. Therefore, it is useful to optimize the template concentration in a PCR reaction to obtain maximum product.

The Primers

Oligonucleotide primers in the range of 18 to 30 bases are generally used for the PCR. The sequence of the primers should be complementary to the 3' and of the target (template) DNA strands to be amplified. Primers are the most important components that determine the success of an amplification reaction. The most important property of a primer is its sequence specificity, which determines what nucleic acid sequence it can bind to, how well it will bind, and how well it will serve as a site for extension of nucleic acid molecules. Generally, a "specific " primer is designed to target a DNA sequence in a closely related group of organisms, while not matching organisms outside that group. Though there are no set rules that will ensure the synthesis of an effective primer pair, the following guidelines are useful.

(a) Wherever possible, select primers with a random base distribution and with a GC content similar to that of the fragment being amplified. Avoid primers with stretches of polypurines, polypyrimidines or other unusual sequences.

(b) Check the primers against each other for complementarity. Use primers with low complementarity to each other. Avoid primers with 3' end overlaps in particular. This will reduce incidence of "primer dimers". Most primers are generally 18 to 30 bases in length and the optimal length to be used in an amplification will vary. Longer primer may be synthesized but are seldom necessary. If shorter primers or degenerate primers are used, the thermal profile should be modified considering the lower stability of the primed target. However, the 3' end of the primer should match the template exactly. Generally, concentrations ranging from 25 to 100 p moles of each primer should be used.

The Reaction Buffer

The components of PCR buffer, particularly the concentration of $MgCl_2$ have a profound effect on the specificity and yield of an amplification product. Concentration of about 1.5 mM is usually optimal (when 200uM each of dNTPs are used). Excess of Mg^{2+} will result in the accumulation of non-specific amplification products and insufficient Mg^{2+} will reduce the yield. Though several buffer formulations have been published, a consensus is beginning to emerge. The recommended PCR buffer should contain 10mM

Tris-HCI (pH 8.4) also. KCl up to 50mM can be included in the reaction mixture to facilitate primer annealing. Excess KCl inhibits Taq polymerase activity.

Gelatin or bovine serum albumin (100 μ mg) and nonionic detergents such as Tween- 20 and NP40 (0.05 - 0.1%) are included to help stabilize the enzyme. The nonionic detergents can be replaced by 0.1% Triton X-100, but some detergent is essential.

Deoxynucleotide triphosphate

The dNTPs are the building blocks of DNA. Once the primer binds to its target site, synthesis of the complementary strand of DNA takes place through primer extension by linking of nucleotide to its 3'end with the help of Taq DNA polymerase. Precursor dNTPs can be obtained as a neutralized solution, which are stable at −20°C for months. The doxynucleotide triphosphate (dATP, dCTP, dGTP and dTTP) is generally used at concentrations of 200 μM each. Higher concentrations may lead to mis-incorporations. Low dNTP concentration reduces mispriming at non-target sites. The lowest dNTP concentration appropriate for the length and composition of the target must be standardized. As a thumb rule, 20μM of each dNTP in a 100ul reaction is sufficient to synthesise 10 pMol of a 400 bp sequence. In the standard reaction, all four triphosphates are added to a final concentration of 0.8mM; this leaves 0.7 mM of the original 1.5mM $MgCl_2$ not complexed with dNTP. Therefore, if dNTP concentration is changed significantly, a compensatory change in $MgCl_2$ may be necessary.

Taq polymerase

The most commonly used thermostable polymerase isolated from the organism *Thermus aquaticus* that is available commercially. Many other thermostable DNA polymerases are also available. The required concentration of Taq DNA polymerase concentration is between 1 and 2.5 units per 100 μl reaction when other parameter are optimum. When optimizing a PCR, enzyme concentration ranging from 0.5 to 5 units/ 100μl are tried and results assayed by agarose gel electrophoresis. If the enzyme concentration is too high, non-specific background products may accumulate and if too low, an insufficient amount of desired product is made.

Thermal Cycles for PCR

Amplification of a target DNA is achieved by repeated cycles of denaturation, primer annealing and extension. These events are controlled by manipulation of temperature. The above three major steps in a PCR are repeated for 25 to 40 cycles. This is done using an automated thermal cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

Denaturation

Double stranded DNA used for the PCR is separated into single strands in the initial denaturation step. Typical denaturation temperature is 94°C for 30 to 60 seconds. Higher temperatures e.g. 97°C may be necessary for G + C rich targets. Denaturation steps that are too 1ong or too high lead to unnecessary loss of enzyme activity.

Primer annealing

At temperatures ranging from 55°C to 72°C, the primers anneal to its complimentary region on the template. The complimentary sequences will form hydrogen bonds between their complimentary bases (G to C, and A to T or U) and form a stable double stranded, anti-parallel molecule. During PCR, the primers are moving around, caused by the Brownian motion in the reaction mix. Hydrogen bonds are constantly formed and broken between the single stranded primer and the single stranded template. The more stable bond lasts a little bit longer (primer that fit exactly) and on that little piece of doubling stranded DNA (template and primer), the polymerase can attach and starts copying the template. Once there are a few bases built in, the hydrogen bond is so strong between the template and the primer that it does not break any more.

The temperature and length of time required for primer annealing depends upon the base composition, length and concentration of the primers. As a rule of the thumb annealing temperature of 5°C below the true Tm of the amplification primers can be attempted. Annealing temperature in the range of 55 to 72°C generally yield the best results. At the optimal primer concentration annealing will require only a few seconds. Increasing the annealing temperature enhances discrimination against incorrectly annealed primers and reduces mis-extension of incorrect nucleotides at the 3' end of the primers. Therefore, stringent annealing temperature, especially during initial few cycles will help to increase specificity.

Primer Extension

The DNA polymerase works ideally at temperature 72°C. The nucleotides (complementary to the template) are linked to the primer on the 3'side by the polymerase, from 5' to 3'; reading the template from 3' to 5' side and bases are added complimentary to the template.

Extension time depends on the length and concentration of the target sequence and upon the temperature. Primer extensions are usually performed at 72°C. The rate of nucleotide incorporation at 72°C vary from 35 to 100 nucleotides per second depending upon the buffer, pH, salt concentration and the nature of the DNA template. An extension time of one minute at 72°C is considered sufficient for products up to 2 Kb in length.

Cycle number

The optimum number of cycles will depend mainly upon the starting concentration of target DNA when other parameters are optimal. Because both strands are copied during PCR, there is exponential increase of the number of copies of the gene. Suppose there is only one copy of the wanted gene before the cycling starts, after one cycle, there will be 2 copies, after two cycles, there will be 4 copies, three cycles will result in 8 copies and so on. Too many cycles may increase the amount and complexity of non-specific background products. Too few cycles give low product yield.

Detection and analysis of PCR product

The PCR product will be a DNA fragments (amplicons) of defined length. The simplest way to check the PCR product is to load a portion of it into an agarose gel containing ethidium bromide along with molecular weight markers and carry out an electrophoresis. The DNA fragments generated by the PCR should be readily visible over an ultraviolet transilluminator. Hybridizing the PCR product with suitable DNA probe is also in practice for conformation.

Nested PCR

Carrying out nested PCR can further enhance the reliability of the PCR. The process utilizes two consecutive PCRs. The first PCR utilizes a pair of primes flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene, which was amplified in the first PCR. The fragment produced by the first reaction is used as the template for the second PCR. Therefore, when information on the sequence of specific genes is available, amplification and visualization of that gene using a nested PCR could be carried out for confirmation.

RAPD-PCR

Arbitrary primed PCR (AP-PCR) / Random amplified polymorphic DNA (RAPD) has been increasingly reported as a method for the genetic characterization. It can be useful for species/strain identification. The underlying theory in AP-PCR/RAPD is that single primer of arbitrary sequence is used that target complimentary sequence on the two DNA strands used as template and amplify the intervening regions in order to generate a genetic profile. The rapid technique, which was developed by Williams *et al.*, (1990) can be used to produce simple and reproducible DNA fingerprints. This is made possible using randomly designed short primers. Genomic variations between and within species could be identified as the difference in the molecular size and number of DNA fragments amplified. The PCR products variations shall be resolved by agarose gel electrophoresis.

PCR- application:

- Site-specific mutagenesis.
- *In situ* PCR: amplification and detection of DNA *in situ* from cells.
- Genomic subtraction.
- Analysis of protein functions and intermolecular assembly.
- DNA fingerprinting using AP-PCR for evaluation of genetic heterogeneity & relationship.
- Generation of single chain antibody fragments by PCR
- Rapid disease diagnosis.
- CDNA synthesis from RNA.
- Production of clones for sequencing.
- Paternity verification
- Molecular taxonomy etc.,

Suggested Reading

- PCR Strategies. Ed.Innis,M.A.,Gelfand,D.H and Sninsky.J.J.,Academy Press, New York.
- PCR - A practical approach. Ed. Mc. Pherson *et al*; Oxford Univ. Press, New York.
- Molecular Methods for virus detection. Ed. Wiedbrauk and Farkas.D.H., Academy Press, New York.

DESIGNING OF PRIMER FOR PCR

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Introduction

The polymerase chain reaction (PCR) is the technique for *in vitro* synthesis of multiple copies of a given DNA molecule. Primers are the most important components of PCR and success of PCR largely depends on the primers. A primer is an oligonucleotide. It is utilized as the starting point for the synthesis of the DNA fragment in a PCR. Primers bind to its complementary sequences on the DNA template and extend in length by addition of nucleotide to its 3' end. Amplification of specific regions of the template DNA depends on the design of primer sequences.

In a PCR, primers are needed as pairs. They are designated as "forward" and "reverse". These primers are complimentary to the 5'ends of regions on the opposite DNA strands which has to be amplified, so that they can be extended toward one another with DNA polymerase , forming new DNA molecules (indicated by the arrows in the diagram).

Since the primers used in a PCR will largely determine the success of the reaction proper attention has to be paid in its designing. There are many factors that guide the primer design .The following points are to be considered while designing a primer.

Sequence specificity

The primers should be specific to the regions flanking the DNA segment to be amplified . The most important requirement of a primer is its sequence specificity. The primer sequence determines what nucleotide sequence it can anneal to, how firmly it will anneal and how good it will serve as a starting point for the addition of new nucleotides to the primers for its extension into new DNA strand. Specific primer are generally , designed to target a DNA sequence flanking the regions to be amplified.

Primer Length

Primer length is an important factor. In general, a primer in the length range of 17 to 30 nucleotides is considered ideal. Longer primer sequences can be used for enhanced specificity in conjunction with higher annealing temperature. The prime requirement of a primer is that it should be complex enough so that the likelihood of it annealing to sequences other than the chosen target is very low.

For example, there is a 1/4 chance (4^{-1}) of finding a single A, G, C, or T nucleotide in any given DNA sequence. Similarly, there is a 1/16 chance of finding any dinucleotide sequence (ie., AG/AC/AT/ etc.) and a 1/256 chance of finding a given tetra nucleotide sequence (ie.,AGCT/ATCC/ etc.). Thus, a sixteen base sequence will statistically be present only once in every 4^{16} bases (Once in every 4294967296, or 4 billion) which is about the size of the human or maize genome. Thus, an oligonucleotide primer of 17 bases or more is extremely sequence specific. Generally, primers of 17 to 30 nucleotides are routinely used for amplification of specific regions of genomic DNA of animals and plants. Extra long primers may result in mismatch pairing and nonspecific priming even at high annealing temperatures. The optimum length of a primer depends on its (A+T) content.

Base composition of primers

The optimum G + C base composition is in the range of 35-60%. It is preferable that the GC content difference between the two primers is within 5%. It is also desirable to avoid long runs of Gs and Cs in primers. Minor adjustments in the lengths of the primers may be made to compensate the differences in them. The G=C pairing is much stronger than the A=T as it has three rather than two hydrogen bonds between them. Hence a GC pair require more heat to melt than the AT pair. High GC content sometimes leads to primer dimer association even after heating to 95°C and as a result in poor amplification of the desired products.

Annealing temperature

The two primers designated as "forward" and "reverse" should have similar melting/ annealing temperatures so they can both work under the same thermal regimes optimally leading to the exponential amplification of DNA in PCR.

As a rule of thumb the following formula is used for determining the Tm values.

$$\mathbf{Tm}\ (\text{degrees C}) = 4 \times (G+C) + 2 \times (A+T) - 5$$

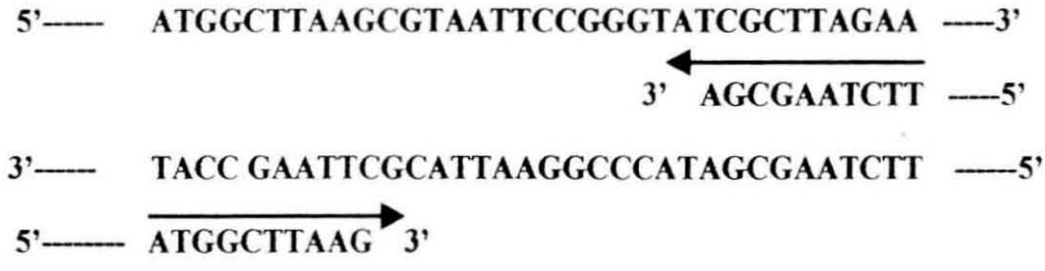
For example the melting temperatures of the following primers work out to be

$$\mathbf{TGGCTTACGAATCGC} \rightarrow 4 \times (9) + 2 \times (7) - 5 = 45°C$$

While designing the primers take care to keep the Tm° of both primers within 1-4°C of each other, and in the 40-65°C range. This formula is applicable only for primers between about 15 and 25 bases length. Other factors like ionic conditions, traces of detergent and solvents, DNA quantity / quality etc.strongly affect actual melting temperature.These factors and time allowed for annealing affect the rate of annealing of primers during PCR.

Primer orientation

If both the forward and reverse primer sequences are designed from the sequence data of coding strand of the DNA molecule, the reverse primer has to match the sequence of the noncoding strand. The reverse primer must be the reverse compliment of the target site of the coding strand from which primer is designed.

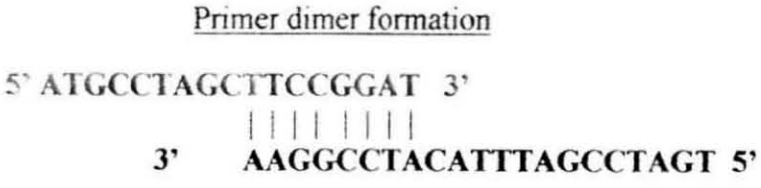


During PCR, the primer pairs bind to opposite strands, and elongation of both primers occur. The forward primer is complimentary to and binds to the non-coding strand, while the reverse primer binds to the coding strand. Not only is the base sequence important, but the 5'to 3' orientation is as well.

Complementarity between primers

The primers should be designed in such a way that there is no complementarity between them. Partial or full complementarity with each other result in the formation of primer dimers (primers binding to each other), thus leaving little of the primers for the priming of the target DNA. Very little primers will be available for target DNA and no/ less amplification product will be formed.

Similarly, care should be taken to avoid complementarity between the 3' ends of the primers. Complementarity between 3' end of primers will also lead to " primer dimer" formation, which inhibits the synthesis of desired PCR fragment.



Self complementarity

In primers of sufficient length reverse-complimentarity between both the ends has also to be avoided especially in long primers as it can loop around and bind to themselves. Like primer dimerisation, this self-annealing will compete with priming of the desired DNA target leading poor amplification.

GC-rich 3′ ends ("GC clamp")

G and C bases have 3 hydrogen bonds and thus bind more strongly than A and T, which share only two hydrogen bonds. Having several G or C at the 3′ end (elongation end) of the primer will make that end more stable and can increase PCR yield.

A/T bases at last codon

Primers should not have T at its 3'end. As T is the least discriminating nucleotide, primers with 3′ T have greater chance of mismatch**.** Further, it is advisable for each primer to have at least one A or T within the last triplet at its 3'to discourage mismatch tolerance of primers with consecutive G's or C's.

5′- End Modification

The PCR primer can work with a poorly matching 5′ end, because the 5′ primer end is not elongated. Therefore, it can accommodate incorrectly matched bases. This is often used for while designing primers for introducing a particular restriction site to facilitate cloning the PCR product.

Primer Degeneracy

When primers are designed from the sequence information of heterologous sources, one cannot often find primer targets that are sufficiently well conserved over a wide enough group of organisms. Thus, we can order a mix of the two (or more) combinations of primer that will cover all sequences. However, as degenerate combinations will greatly increase the chance of priming of an undesirable sequence unrelated to your sequence of interest.

Primer designing guidelines in brief:

1. Primers should be 17 to 30 nucleotides in length.
2. Base composition should be 35-60% (G+C)
3. The 3′ end of primers should have preferably a G or C, or GC or CG.
4. Tm values of primers between 55-65° C are preferable.
5. Runs of three or more Cs or Gs at the 3′ ends of primers should be avoided.
6. Primer orientation should be correct while designing.
7. The 3′- ends of primers should not be complementary to each other.
8. Self-complementarity of primers should be avoided.

Assistance for Primer designing

The primer sequence should be specific to the regions flanking the DNA segment to be amplified. Sequence specificity can be worked out and checked using software programmes. Database which provide sequence information for regions of interest (NCBI: ENTREZ) can be utilized for this. Comparison of sequences can be made with large number of sequences stored in database and similarities can be checked by NCBI: BLAST programme. Many soft wares are available on Internet for designing of primers of a desired sequence.

Internet site(s)

http://biobase.dk/index.html

http://bcf.drl.arizona.edu/gcg.html

http://www.blocks.fhcrc.org/

http://www.biodisk.com/

http://www.oligo.net

http://www.hgmp.mrc.ac.uk/

http://bioinformatics.weizman.ac.il/blocks/index.html

http://alces.med.umn.edu/webprimers.html

http://www.willamstone.com/

http://doprimer.interactiva.de/

http://www.genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi

http://dot.imgen.bcm.tmc.edu:9331/seq_util/seq_util.html

http://www.med.jhu.edu/medcenter/primer/primer.cgi

Procedure

1. Search National center for Biotechnology Information (NCBI= 'gene bank') for selected /assigned type of sequence / gene family, using the 'Entrez Browser' program. Try various keywords and ' wildcard' search combinations to try to find all known entries of selected sequences/gene family. Also use the "taxonomy" browser to find closely related species.
2. Download the sequence files and clearly label these files

3. Prepare a text file containing only the sequences

4. Remove the nucleotide numbers from the sequence.

5. Submit the edited sequences to the primer 3 server, by pasting the edited sequences into the submission window.

5. Make entries in the parameters boxes such as sequence ID, target sequence and length of amplified product.

7. For initial stages of primer designing, leave other parameters at default settings

8. Run the programme. The output will give several combinations of primer pairs for a particular target sequence. Primer 3 gives the sequences of primers- forward and reverse in their proper orientation.

9.The primers are then tested for the secondary structure formations in software in which this facility is available e.g. DNASIS.

QUANTITATIVE GENETIC TOOLS FOR BROOD STOCK IMPROVEMENT

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Introduction

Success in aquaculture and fisheries management depends on viable concept, sound management and animals with high genetic potential. In farm animals and plants application of genetic principles and genetic tools lead to increased production in accordance with the demands of the nation. Breeding programmes like selective breeding and cross breeding played an important role in increased productivity, domestication and survival rate. Genetics in fisheries is relatively new. Of late genetics has acquired an important place in fisheries with the following objectives.

1. Production of high yielding strains of fish and shellfish.
2. Development of disease resistant strains.
3. Conservation and management of natural resources.

Gregor John Mendel, father of genetics postulated laws of inheritance in 1865 by conducting his experiments on pea plants. He was successful because he ensured that the plant bred true for a particular trait and selected simple, easily recognizable and highly heritable characters. He suggested that corresponding pairs of factors in the parents control the pairs of alternative characters. The offspring was supposed to have one of each factors contributed by each parent. Subsequently, those factors were called as genes by Batsen and their physical nature and chemical properties are established. The theory of inheritance provided the mathematical basis for predicting results of mating and testing the laws in animals and plants. Quantitative/ Population genetics is the logical development for the basic principles of inheritance. Quantitative genetics is the theoretical basis for all the breeding programmes in animals and plants. Continuous variation characterizes the most of the economic traits. These traits are called quantitative traits, which are controlled by many genes called minor genes. In contrast qualitative traits show discrete variation and controlled by few genes called major genes.

Tools available to the breeder for improvement

Variation

Variation among the population is the basic and most important pre- requisite for any genetic improvement programme. With out variation there is no improvement. Variation is defined as the mean of the squared deviations from the population mean. Variation is expressed in terms of Variance (σ^2).

In order to study the genetic properties of the population we have to partition the phenotypic variation in to component parts attributable to different causes. The phenotypic variation can be divided in to components attributable to the influence of genotype and environment. The genotype is the particular assemblage of genes possessed by the individual and the environment is the non-genetic effects. The two components associated with the genotype and environment is the genotypic variance and environmental variance.

$$V_P = V_G + V_E + V_{G-E}$$

Where,

V_G = Genotypic variance

V_E = Environmental variance

V_{G-E} = Genotype and environmental interaction

The partition of the variance in to its components formulates the question of importance of individual portion in determining its phenotype. The relative importance of cause of variation means the amount of variation it contributes to the total variation. So the relative importance genotype as the determinant of phenotype is the ratio of genotypic variance to phenotypic variance (V_G / V_P). This is termed as the heritability (h^2).

Genotypic variance can be further divided in to additive genetic variance (breeding value) and non-additive genetic variance (Dominance and interaction deviations).

$$V_G = V_A + V_D + V_I$$

Where,

V_A = Additive genetic variance

V_D = Dominance deviations

V_I = Epistatic interactions

Additive genetic variance: It is the sum of the effects of all the alleles that help to produce a phenotype. It is the variance of the breeding values. It is the important component because it is the chief cause of resemblances between the relatives. It defines the genetic properties and characters of the population. This portion of the variance is transmitted to the offsprings from the parents.

Non-additive genetic variance: These variations are due to the dominance and epistatic effects of the alleles, viz.,

Dominance deviations: This is due to the dominance effects of the alleles at the loci. V_D is not transmitted to the next generation from the parents since the alleles are disrupted during the meiosis.

Interaction (Epistasis) variation: It occurs due to the interaction of the alleles between two or more genes. These interactions may be two factor or three factor or so on. The amount of variation due to interaction is rather small; some times the breeder can omit it also.

Environmental variance: It is the variance due to all non-genetic effects starting from feeding to environmental conditions. Environmental variance cannot be eliminated in a population. It plays a major role in determining the phenotypic variance of the population.

Genotype and environmental interaction: Apart from the above two components of variation there is interaction between the two components. That is a genotypic effect of the population is different in two different environments. A single genotype may perform differently in two different environments. This genotype may show good performance in one Environment but may be poor in second. Other one is the performance of the populations may be reduced or increased in degree of magnitude in different environments.

Heritability (h^2)

The heritability of the metric character is one of the most important properties of the economic traits. It expresses the proportion of the total variance attributable to genetic causes, which determines the degree of resemblance between relatives. But the important function of heritability in quantitative genetics/Animal or plant breeding is its predictive role in expressing the reliability of the phenotypic value as a guide to the breeding value.

The heritability is defined as the ratio of genetic variance to phenotypic variance in a broader sense.

$$\text{Heritability } (h^2) = \frac{\text{Genotypic Variance}}{\text{Phenotypic Variance}}$$

$$h^2 = V_G / V_P$$

It can be defined as the ratio of additive genetic variance to the phenotypic variance in narrow sense.

Heritability (h^2) = $\frac{\text{Additive genetic Variance}}{\text{Phenotypic Variance}}$

$$h^2 = V_A / V_P$$

Regression of breeding values on phenotypic value is the heritability,

$$h^2 = b_{AP}$$

Estimation of heritability

Method of estimation	**Covariance**	**Regression/ Correlation**	**h^2**
Half sib mating	$1/4\ V_A$	$t = 1/4\ h^2$	4t
Full sib mating	$1/2\ V_A + 1/4\ V_D + V_{EC}$	$t = 1/2\ h^2$	2t
Offspring one parent regression	$1/2\ V_A$	$b = 1/2\ h^2$	2b
Offspring mid parent regression	$1/2\ V_A$	$b = h^2$	b

Heritability ranges from 0-1 or expressed in percentage. h^2, plays an important role in selection programmes for genetic improvement. It is the property of the population not of an individual for particular trait under selection. Thus it is valid for only given population in a given environment. It is useful in predicting the impact of selective breeding.

Genetic correlations: Economic traits are correlated; means selection for one trait may lead to increase or decrease in the other traits. These are called correlated responses and the traits are called correlated traits. This is expressed in terms of correlation co-efficient (r), which ranges from -1 to +1. Correlated response may be positive or negative. r, is due to the pleotrophic effect of the alleles. This plays an important role in trait selection for improvement.

Selective Breeding for fish stock improvement

Breeding is the practical aspect of the genetics, clear and thorough understanding of the genetic rationale and genetic principles will avoid the in advent mistakes during the breeding. So before planning a breeding programme breeder should have clear understanding of its pedigree records, genetic parameters like heritability, correlations and of course reproductive biology and behavior of the species.

Selective breeding means careful selection of superior performing individuals in a population as parents for the next generation. This is based on the Robert Backwell theory of

"like begets like". Two elite/best performing individuals are selected and bred to produce the best performing progeny. In animals and plants this lead to production of pure strains and also to improve the production. Selective breeding of fishes started in recent years only. This has been useful approach for genetic improvement of cultured fish populations. However the breeder should well aware of the associated problems of selective breeding programme, viz.,

1. Inbreeding depression
2. It is time consuming and expensive
3. Selection of the superior animals is very cumbersome and difficult.

The selective breeding involves the following steps.

Selection of species

Selection of the species is very important aspect of selective breeding programme. While selecting a species the breeder should consider two things, the first is it should have well established breeding and seed production technology and the second is its economic importance.

E.g.: Catla –is a good species for selective breeding

Selection of trait

The trait or traits under selection should be economically important, highly heritable, positively correlated and it should be easily measurable. Some economically important traits in fishes are growth rate, body size & shape, meat quality and disease resistant.

Methods of selection

Selection of the parents for next generation is based on its additive genetic variance or breeding value. The genetic potential of an individual is judged by the phenotypic performance of the individual in several ways.

Individual selection

The individuals are selected based on their own performance, on their phenotypic value. This selection method is very effective when the h^2 of the trait is high. It is usually simple to operate and it yields the rapid response. Mass selection is the term used for individual selection when selected animals are kept together *en mass* for mating.

Family selection

Families are selected or rejected as a unit according to the mean phenotypic value of the family. In this within the family variations are not considered. The accuracy of the family selection depends on the heritability of the trait, family size, family type and the variation due to the environment. This is more useful when selection is practiced for less heritable traits like reproductive traits, carcass quality and disease resistant.

Pedigree selection

In this method individuals are selected based on the performance of their parents or grand parents. Pedigree records of the ancestors considered for selection of parents.

With in family selection

In this method family is considered as a sub population and individuals are selected based on the performance in relation to the mean performance. Better performing individuals from the family are retained and the others are culled. This method is more efficient than individual and family selection. Selection within families would eliminate the non-genetic variation.

Progeny testing

Parents are selected based on the performance of the progeny. This method is reliable and best in farm animals; in fishes it is not suitable when we are using cryopreserved gametes during the selective breeding programme.

Tandem selection

In this method of selection one trait is considered at one time. The selection is aimed at improving one trait at a time for generations till the goal is reached than other trait is selected and it continued for all the traits. It is simple but time consuming, it is inefficient because it takes long time and there is a chance of negative correlation, which decreases the mean performance.

Millenbach (1950), Selection for age at maturation at 2 years instead of 3 and 4 in rainbow trout lead to poor growth rate and less egg production. Ehlinger (1977) brook trout selected for increased resistance to furunculosis had become more susceptible to gill disease.

Independent culling levels

Independent culling level is a selection programme employed when two or more traits are considered for selection simultaneously. In this the breeder has to fix the minimum performance level for each trait, and the fish must perform above the minimum level. The disadvantage of this method is a fish should be outstanding in all the traits under selection.

A fish with best performance in one trait and average performance in the other is liable for rejection. One more disadvantage is when selection is practiced for more traits at a time you may end up with few individuals, which satisfy the minimal performance levels of all the traits.

Selection index

Index selection is the best and most efficient method of selection progrmme. In this method all the traits considered for selection are given due weightage according to their performance and a selection index is formulated based on the linear regression. In this all traits are given importance.

$$I = b1X2 + b2X2 + \ldots\ldots\ldots\ldots + bnXn$$

Where,

I	Selection index
b	regression coefficient
X	Phenotypic value of the traits

Breeding plan

Selective breeding programme intends to exploit the existing natural population, so there should be vide genetic base in the base population. For this different stocks/animals of the species are selected from different areas/geographical regions and make sure about the presence wide range of germplasm. Before introducing the animals in to the breeding they should be evaluated and studied thoroughly with regard to genetic variation, heritability and genetic correlations. These parameters will help the breeder to visualize the success of the breeding programme.

Selective breeding is a type of inbreeding/pure breeding in which superior individuals from the population are selected and bred to produce the next generation. Continuous selection eliminates the deleterious recessive alleles present in the population. It makes the population complete homozygous. This breeding method is very much useful in evolution of different strains and lines.

Crossbreeding

During the selective breeding breeding there is always chance of inbreeding depression, which a breeder should concern about. To overcome this crossbreeding between the strains or inbred lines is employed. Out crossing is very useful method in selective breeding where in unrelated individuals of same species are bred to minimize the effect of

inbreeding depression. Crossing of two inbred lines increases the performance because of heterosis. Heterosis is the observed superiority of the parents from the population mean.

Hybridization

It is the interspecific crossing of the fish. Hybridization is the rapid method of bringing about genetic modification.

Evaluation of the response for selective breeding

The genetic gain from the selective breeding programme can be evaluated by calculating the following.

i. Heritability
ii. Selection differential
iii. Generation interval

Heritability (h^2): It is the important factor that determines the genetic progress after selection. h^2 varies from population to population and environment to environment. Thus the h^2 estimated under experimental conditions may not be same under practical conditions.

Selection differential (S): The difference between the mean phenotypic value of the population and mean phenotypic value of the selected individuals is called selection differential. The selection differential is more if there is more variation among the population because only superior animals were selected for next generation. So the selection intensity is high. Selection intensity can be calculated

SI = Selection differential/Standard deviation of the phenotype

Response to selection (R): It is the difference of mean phenotypic value between the offsprings of the selected parents and whole of parental generation before selection. It is average superiority of the selected parents.

$R = h^2 S$

Generation interval: It is defined as the age of the parents when their offsprings are born. It is the interval between the two generations. It varies between the species.

Examples: Salmon and Catla - 2-3 years
Zebra fish - 3 months

The expected response for generation is the product of the selection differential and heritability per generation.

Genetic gain per year(R) = $h^2 S/GI$

$$= \frac{h^2 \times Six\ sd}{GI}$$

During the evaluation of the selection response, the breeder should take a note whether it has any parallel effect on the traits other than the one selected in the breeding programme. The positive or negative correlation of other traits is called *correlated response.* For example, increased fecundity, fry survival and disease resistance were the correlated responses to selection for increased body weight in Channel catfish. On the other hand, selection of body weight did not affect the spawning, hatchability of eggs, fry survival and seinability, so they are negatively correlated (Smitherman and Dunham, 1985).

Impacts of selective breeding programme on aquaculture productivity.

Selective breeding has been found to be a very useful approach for improving the qualitative traits like the attractive colouration pattern in the ornamental fishes and the commercially important quantitative traits in food fishes. This has helped to improve the aquaculture productivity of Atlantic salmon in Norway, Channel catfish in USA and Tilapia (*O. niloticus*) in Philippines. The body weight of *O. niloticus* and Channel catfish increased by about 15% by mass selection. (Bondrai *et al.,* 1983; Smitherman and Dunham, 1985). Six generations of selection has increased the body weight by 30% in Rainbow trout. In Coho salmon, 50% increase in growth rate was observed after 10 generations of selection (Hershberger *et al.,* 1990). Selective breeding of *Labeo rohita* for improving the growth rate is being carried out at Central Institute of Freshwater Aquaculture (CIFA), Bhubaneswar, India. Faster growing seeds have been distributed on a limited scale for culture. Selection has also helped to improve disease resistance ability in some fishes like resistance to bacterial disease dropsy in Common carp (Kirpichinikov *et al.,* 1993). Newkirk and Haley (1983) conducted selective breeding experiments for increased growth rate in European oyster, *Ostrea edulis* and found that the offsprings of selected group were heavier than the controls and the rate of survivability was higher. Mallet and Haley (1983) studied the inbreeding effect on larval and spat performance in the American oyster, *Crassostrea virginica* and reported that there was an increased survival rate in inbred families but there was decrease in shell surface area.

Oyster breeding programs to select for fast growth in Sydney rock oysters in NSW and Pacific oyster in Tasmania have been successful in increasing the growth rate (Nell et al., 1999). Mortality rate from QX disease was reduced in progeny of a second generation disease resistant line as compound with controls (Nell and Hand, in press). Triploids in pacific oyster have been successful in commercial level in North America, Europe and Australia. (Nell, 2002)

However, the selection for the higher growth rate was either unsuccessful or had some negative effects in certain cases. Five generations of selection for increasing the

growth rate in Common carp in Israel had led to decrease in the body size (Moav and Wolhforth, 1976). Similar effect was also observed in case of *O. niloticus*.

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SELECTIVE BREEDING FOR GENETIC IMPROVEMENT OF BROOD STOCK

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Introduction

Though one can bring about improvement in the production performance of a population by environmental manipulations as well as through genetic manipulations, any improvement from the former cannot be transmitted to the next generation. The genetic improvement on the other hand is inherited by the next generation and therefore, more important. The wide variations among the individuals in every population provide ample scope for genetic manipulations for developing genetically superior lines and strains.

A number of modern genetic manipulations techniques like chromosomal engineering for production of polyploid, gynogenic and androgenic populations as well as genetic engineering, where a desirable gene or set of genes from one animal can transferred into another for producing a transgenic animal with desired characteristics are available for genetic improvement. However, the conventional quantitative genetic techniques like selective breeding can be an attractive technique for production of genetically superior brood stock.

Selective breeding has been used for thousands of years to improve all major crops and livestock. Selective breeding, as used in farm animals and crops, is the time-tested genetic manipulation technique, which can play a definite major role in developing genetically improved fish/shellfish also. However, an in-depth knowledge of the genetics of the animals is a pre-requisite for the formulation of the appropriate techniques for their improvement. Breeding is the applied science of genetics. Inbreeding is often combined with hybridisation to improve the results of the crossbreeding programme. To date, much of the selective breeding work in aquaculture has been devoted to Atlantic salmon, Channel catfish, Rainbow trout and Tilapia. Though, there have been some work on the selective breeding of Carps in our country, it still remains a grey area with much to be done.

Very few attempts have been made for the genetic improvement in mariculture species through selective breeding for enhanced growth as disease resistance, the shrimps being no exception. The main reason is that they are either not domesticated or at the very early stages of domestication. However, of late scientists are successfully, breeding and rearing shrimps under captivity paving the way for selective breeding endeavours. For most aquaculture species, selective breeding is difficult became the juvenile are too small to be tagged for later identification and growth evaluation. Frequent moulting of the shrimps compounds the problem.

Use of selective breeding and marker-assisted selection has been recently introduced in crustacean improvement programmes. Scientists of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia are undertaking programmes for genetic improvement of *P.japonicus* and have reported good response.

The ultimate goal of every selective breeding programme is to improve the breeding value of the population. To accomplish this goal, a breeder selects (saves) those individuals that possess certain desired phenotypes and culls (removes) those that do not. By selecting and mating only the best ones he hopes that the selected brood will be able to transmit their superiority to their offspring, thereby creating a genetically improved population. If this occurs, the next generation will be more valuable and will increase their market value.

Selection is the process in which certain individuals in a population are preferred over others to produce the next generation. Natural Selection is exercised by nature. Survival of the fittest is the main force responsible for selection by nature. Artificial Selection is the one practiced by man, in his effort to increase frequency of desirable genes in the stock. Selection does not create new genes, but increase the frequency of desirable genes in the population leading to increased proportion of homozygous individuals. Selection is classified on the basis of selection criteria as follows.

1) Individual Selection : Selection of the breeders based solely on the individuals own phenotypic performance i.e., phenotype is used as the indicator of his genotype.

2) Family selection: Family consisting full-sibs and /or half sibs is the unit of selection and the decision to save is based on family mean.

3) Pedigree Selection: Selection based on ancestor's performance (i.e. using performance pedigree) Pedigree is the record of individual's ancestors related it through its parents.

4) Selection on the basis of progeny testing: Selection of breeders based on the average merit of the offsprings as compared to the average merit of the progeny of contemporary breeders. Generally used for selecting sites in terrestrial animals. But in fish it can be used for even dams.

Genetic gain in quantitative trait from one generation of selection

$$\Delta G = h^2 \times Sd; \quad \text{where } Sd = (\overline{P_s} - \overline{P})$$

Yearly generic gain : $\Delta_{Gt} = \frac{h^2 \times Sd}{I_g}$

Knowledge about the phenotypic and genotypic parameters of the economically important traits is vital. Estimates of parameters such as genetic variation, heritability, phenotypic and genetic correlations, heterosis, genotype environment interactions etc are essential for planning a proper breeding strategy. Therefore, breeding studies for the evaluation of the genetic and phenotypic parameters are the essential pre-mediated step. Decision about the organisation of breeding programme could be made only after careful consideration of the above parameters. As for example, when there is relatively larger additive genetic variance, simple selection methods like individual/mass selection should yield good progress. On the other hand if nonadditive genetic variance is predominant, special selective breeding schemes are to be formulated to exploit them. When over dominance is important for a trait, reciprocal or recurrent reciprocal selection needs to be employed. If heterosis is found to be high, cross breeding programmes could be given priority. Genotype environmental interactions of high magnitude call for developing different strains to suit each of the environments.

Both quantitative and molecular genetic tools can be applied in conjunction for improvement of mariculture species. Of late the use of marker assisted selective breeding is being attempted. Combined application of quantitative genetic techniques and molecular level markers can pay rich dividends in the future.

The progress in cultured fish and shrimp genetic research has been slow due to the lack of sufficient knowledge on the basic aspects of their biology. Of late, some of the genes involved in growth reproduction and disease resistance of shellfishes have been identified, cloned and sequenced. The scientists CSIRO, Australia have reportedly produced genetically improved *P.japonicus* which matures three weeks earlier and grows 25% more than the unimproved cultured stock.

The salient results of a selective breeding programme carried out in artemia at CMFRI, Cochin to study the quantitative genetic parameters as well as the response to selection as part of the doctoral programme are presented briefly below.

Materials and methodology:

Artemia franciscana (Kellogs1906) from Great Salt Lake, Utah was used for the study. Method of selection followed was Mass selection (individual selection) and the trait under selection was the naupilar size (length in µm). Bi-directional mass selection was practised in two sub-populations derived from the base generation viz., SNS line & BNS line with the aim of developing two divergent stocks. While selection for reducing the naupliar size was practised in SNS line, BNS was selected for bigger naupliar size. Six selected generations were raised. Intensity of selection (i) common for male and female together was estimated as the mean of two sexes i.e. $i = \frac{1}{2}$ (im + if) (Falconer, 1981). Intensity of selection for male and female separately calculated as ratio of effective selection differential to the phenotypic standard deviation. The heritability values of the selected trait (naupliar size)

were estimated from full sib data and from regression of offspring on parent as per the procedure given by Becker (1975).The predicted genetic response per generation was calculated for each line separately within sex as per the procedure described by Falconer (1960).

Results :

Heritability of naupilar length:

The heritability estimates of naupliar length, from the regression of progeny on parents, pooled over generations, were 0.2123 ± 0.0766 and 0.3885 ± 0.1108 for males and females respectively in SNS line. The corresponding estimates in BNS were 0.5777 ± 0.1154 and 0.3364 ± 0.1176 respectively. The heritability estimates from full sib data, pooled over generations, were 1.3256 ± 0.0474 and 1.1004 ± 0.0522 for males and females respectively in SNS line, whereas, the corresponding estimates in BNS were 1.2580 ± 0.0583 and 1.4221 ± 0.0479 respectively. While the moderate values of heritability estimated from b_{op} indicated existence of fairly good amount of additive genetic variance which can be exploited through simple selective breeding techniques, the very high estimates of heritability from full sib analysis indicated existence of non-additive genetic variances also.

Selection differential:

Selection differentials, averaged over generations, were slightly higher in females of both SNS and BNS lines. Their mean values were -16.6780 μ m and -16. 3966 μ min SNS males, -19.9266 μ m and -22.3101 μ m in SNS females, 16.2308 μ m and 15.8700 μ m in BNS males and 17.1180 μ m and 17.0019 μ m in BNS females.

Phenotypic responses :

Phenotypic responses for naupliar length from selection were quite substantial. The naupliar size in SNS line, from six generations of selection for smaller size, could be reduced from 486.99 μm and 490.58 μm in males and females respectively to 441.67 μm and 453.05 μm. The cumulative gain for males and females were -44.32 μm and -37.52 μm respectively with average gain per generation being -5.76 μm and -4.96 μm. In the BNS line, the naupliar size could be increased to 495.75 μm and 529.37 μm in males and females from 486.99 μm and 490.58 μm in the base generation, through five generations of selection for bigger naupliar size. The total gain worked out in males and females were 8.59 μm and 38.80 μm with mean gain of 0.39 μm and 5.52 μm respectively. The mean phenotypic responses were statistically significant except for BNS males.

Realised genetic gain:

The observed phenotypic response is the combined effect of both genetic and environmental factors. Since the environment rarely remains the same over the period of selection, separating out these effects becomes rather difficult. One of the most commonly used methods for removing environmental effect from the phenotypic gains and for

determining genetic gain is the use of an unselected control population, preferably, from the same stock as that of the selected population. Such a control line was used in the present study. Most of the phenotypic responses realized from selection were due to genetic gains. In the SNS line, total genetic gain realized from six generations of individual selection for reduction of the naupliar length was -41.7244 µm in males and -38.7585 µm in females. Whereas in BNS line, the total genetic gain from five generations of selection were 12.6427 µm and 39.4836 µm in males and females respectively.

The realized mean genetic gain per generation, estimated from regression of control corrected generation means on generation numbers was -5.2585 µm in males and -5.2289 µm in females of SNS, and 0.9338 µm in males and 5.3493 µm in females of BNS line. The mean genetic gains were fairly high and statistically significant except in BNS males.

Expected genetic gains :

Expected responses were calculated using heritability estimated from regression of offspring on parent (bop) and also full sib heritability. While, estimates as per former were close to realized genetic gains, those from latter were on the higher side. This result indicates that heritability estimates from full sibs are indeed inflated by non-additive genetic variance, unlike the bop, which includes only additive genetic variance.

Generation wise phenotypic response in naupliar length realized from bi-directional selection for reducing naupliar length in SNS line and for increasing naupliar length in BNS line showed that the response in both the lines were in the desired direction. The total cumulative decrease in naupliar length from six generations of selection for smaller naupliar size viz. -45.3177 µm and -37.5220 µm in males and females respectively, works out to 9.3057% and 7.6486% of the naupliar size of the base population. Similarly, cumulative increase of 8.5923 µm and 38.7966 µm in males and females of BNS line from five generations of selection for larger naupliar size work out out to be 1.7644% and 7.9084% of the naupliar size in base population. The mean decrease in the naupliar length per generation in SNS line was -5.7554 µm and -4.9743 µm for males and females respectively. The corresponding increase per generation in BNS line was 0.3833 µm and 5.5222 µm respectively. It can thus be seen that while in SNS line both the sexes readily responded to selection for decrease in naupliar size, there was a differential response to selection for decrease in naupliar size there was a differential response to selection for the higher size in BNS line. In BNS line, the females showed 14.5 times higher response than males, while in the SNS line both sexes showed comparable response. It is rather difficult to explain whether this low response in males was due to attainment of the genetically pre-set maximum size for that sex or due to any other reasons.

The point to be noted in this context is that the male nauplii were always smaller than females in both SNS and BNS lines as well as in the base population. The smaller size

of males as compared to females may be the nature's provision to enable them to clasp the female quite easily and to maintain buoyancy during copulation. The males might have reached the size limit set by nature and hence have exhibited a lower response, when larger sized nauplii were selected.

The realized response calculated by subtracting the mean control values of each generation from the corresponding selected generation mean is free of environmental effects and therefore, gives the true genetic gain from selection. Comparison of the genetic and phenotypic gains realized in this study point towards the fact that though the environment had played a role in deviating the phenotypic response from the genetic response, its effect was comparatively low and that the genetic gain was quite substantial.

Most of the documented selection studies in the aquatic species have reported the response to selection with out considering the environmental effects and therefore, represents only the phenotypic response and not the genetic response. In this study, response to bi-directional selection was in the expected direction, though the rate of response was relatively of higher magnitude in line selected for reduction of naupliar size. The substantial genetic gains realized from selection indicate the usefulness of selective breeding for developing genetically altered lines. Very few bi-directional selection studies, with reference to growth, have been reported in aquatic animals. While Moav and Wahlforth (1976) observed no response from five generation of selection for high growth rate in common carp, there was a strong response to selection for slow growth rate. In channel cat fish, Bondari (1983) reported response to selection for body weight and length in both upward and down ward directions. Huang and Liao (1990) reported little response to mass selection for high body weight as well as for high body weight as well as for low body weight in tilapia. Behrends et al. (1987) and Rochetta (1996) could not observe any response to selection for growth in tilapia and guppy, due to the prolonged domestication process in these fishes. No comparable results from *A.franciscana* are available.

Genetic improvement need not be restricted to maximising growth and feed conversion, but can be in survivability and disease resistance. Susceptibility to disease especially viral infections is playing havoc with the shrimp culture and needs immediate attention. Like any other biological traits there is naturally occurring genetic diversity in disease resistance also, leading to differential susceptibility of individuals of a population to various pathogens, leading to the survival of some individuals even after a disease out break. Selective breeding for disease resistance, supported by marker-assisted selection is need of the hour, which calls for investigations in the field of genetic markers of enhanced disease resistance.

The decision to conduct a selective breeding programme is a decision that must be made on a case-by-case basis. Because selective breeding programmes require dedication, a certain level of sophistication, record keeping, and the investment of extra labour. Additionally, selective breeding programmes are not free; they also require the investment of

money. Finally, these programmes usually do not produce immediate improvements. Improvements are usually not seen for at least one growing season, so a breeder must be able to incorporate long-term planning into his programme, and he must be patient. A final requirement that must be met for conducting a selective breeding programme is the existence of proper facilities.

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GYNOGENESIS AND ANDROGENESIS IN FISH

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Gynogenesis

Gynogenesis is a developmental process facilitating the inheritance of maternal genetic material alone. Induction of gynogens involves two steps: 1. Elimination of paternal chromosome, i.e. activation of egg with irradiated homologous sperm, or intact or irradiated heterologous sperm, and 2. Restoration of diploidy by a shock to retain the second polar body, or suppress the first mitotic cleavage. Gamma and X-rays have high penetrance inducing chromosome fragmentation. Therefore, larger volume of sperm can be irradiated. UV light damages chromosomes by inducing thymidine dimers, which may be repaired by photo-reactivation process. The low penetrance of UV light permits the treatment of diluted (4 to 100 times), smaller volume (~ 2.5 ml) of sperm standing to a maximum height of 2 mm. Gamma and UV-treated sperm may still transfer a fraction of genetic material to putative gynogens.

Gynogenesis occurs naturally in some fish species belonging to the family Poecilidae. A gynogenetic species consists only of female individuals. The females mate with the males of the related species to acquire sperm, which is needed to initiate embryonic development. Gynogenesis was induced artificially in a frog for the first time by Hertwiz in 1911. He fertilized the frog's eggs in several batches with its sperms treated at different doses of radium irradiation. Surprisingly, the sperms irradiated at low dose caused lethality but the sperms irradiated at high doe produced viable offspring. This paradoxical phenomenon is called *Hertwig effect.* At a low dose of radiation, the embryos died due to genetic abnormalities. At higher does, the sperm chromatin gets completely inactivated but it can trigger embryonic development leading to the production of haploid embryos. The survival rate of haploid embryos is very low, which can be improved by inducing diploidy.

Many authors were cautious to identify "genetically impure gynogens" using one or other morphological character as a marker like colour, scale pattern etc to trace paternal contamination. Quillet & Gaignon (1990) Castelli (1994) have chosen sperm from a donor species, which will not yield hybrid or triploid; for instance, sperm of *Oncorhynchus mykiss* do not produce hybrid or triploid, when crossed with *Salmo salar;* sperm of *Cyprinus carpio* crossed with *Barbus barbus.*

UV radiation is known to result in the production of cyclobutyl rings between adjacent pyrimidines (mostly thymine) on the same DNA strand. These dimers can be split *in situ* by a specific light absorbing enzymatic repair system (photolyase), resulting in the photolysis of the cyclobutyl rings. Hence, this process of enzymic photoreactivation

can restore the mono pyrimidines. To prevent photo reactivation of the inactivated chromosomes of sperm, the entire process of sperm irradiation and activation condition of fish has to be conducted under complete darkness. Yet, the sperm have been shown not to contain the photoreactivation enzyme in the catfish *Rhamdia sapo.* However most authors were doubly cautious to choose suitable heterologous sperm and irradiate them; yet egg activation by irradiated homologous or heterologous or intact heterologous sperm inherently holds the scope for paternal genetic contamination; in fact such genetic contamination, estimated from colour marker, ranges from 0.006 % for ^{60}Co-gamma-irradiated sperm to 0.03 % for UV- irradiated sperm.

Diploidization is usually achieved by shocking the activated eggs; the effective pr[illegible] summarized. Two kinds of gynogenesis are possible: "meiotic gynogenesis" in which the second polar body is retained, and "mitotic gynogenesis", in which the first mitotic division of zygote is suppressed. Retention of the second polar body permits effective duplication of the genome without interfering the crossing-over events, but will guarantee some levels of heterozygosity. Therefore, the products of meiotic and mitotic gynogenesis are heterozygous and homozygous gynogens, respectively. Consequently, meiotic gynogenesis can produce isogenic lines of fish within a few gynogenetic generations, but not homozygous (clonal) lines of fish; homozygous (clonal) lines of fish can be established by mitotic gynogenesis in two successive generations. Therefore, research in gynogenesis may help to (i) produce all-female populations, (ii) establish isogenic and homozygous lines of fish (iii) increase higher production atleast in some species and (iv) understand the genetic regulation of development and construction of linkage maps.

Use of gynogenesis for chromosome mapping is currently one of the most useful functions. The principal benefit of meiotic gynogenesis in applied breeding now is its usefulness as an experimental approach to define methods for induced polyploidy.

Androgenesis

Androgenesis is a developmental process facilitating the inheritance of paternal genetic material. It involves inactivation of the egg's genome, and dispermic or monospermic activation by haploid or diploid gamete. It was doubted the scope for total elimination of egg's genome, since mitochondrial DNA and messenger RNA are present in large quantities in the egg. Owing to the protection afforded by the mitochondrial membrane, mitochondrial DNA present in eggs of *Oreochromis niloticus* suffered no damage from UV- irradiation; yet it is considered that the radiation may disrupt maternal RNA and membranous structures in the egg and thereby the fate of individual cell and cell lineage, may affect the differentiation process leading to deformed hatchling. Notably, the intensity of radiation, to which the sperms are subjected, is generally less than 10 % of the UV dose administered in eliminating the egg genome. Masaoka *et al.* (1995) have recorded upto 10 % maternal genomic contamination in the androgenetic *Misgumus anguillicaudatus* using genetic markers. The NBFGR, Lucknow has successfully produced live diploid common carp androgen, but did not use genetic markers to confirm 100 % elimination of maternal genetic contamination.

Androgenesis may prove useful technique for production of (i) viable (YY) supermale in male-heterogametic species, (ii) inbred isogenic lines and (iii) conservation of germplasm. When crossed with normal female, supermale can produce all-male progenies. By hormonal sex reversal, it has also been possible to produce (YY) superfemales in oviparous (e.g. *Oreochromis niloticus)* and viviparous *(Poecilia reticulata)* species. Viable supermales have been produced *(Oryzias latipes; Oreochromis mossambicus;* O. *niloticus; Poecilia reticulata*) by combining hormonal sex reversal and gynogenetic technique; Of course, inviability of YY male has also been reported (e.g. *Betta splendens).* Androgenesis has successfully induced viable YY males in a few cyprinids, cichlids and salmonids.

Considering the immense potential for the production of new strains of economically important and other species, there is an urgent need to develop new technique for conservation of wild strains of fish. Because of the telolecithal nature of fish egg, cryopreservation of embryos and eggs remain unsuccessful until today but, the cryopreservation of sperm has been quite successful. Being amenable for cryopreservation and induction of androgenesis, fish are uniquely advantageous, as these techniques can profitably be used to restore a strain or species from the cryopreserved sperm. Indeed cryopreserved sperm may become' the gene banks' for the wild strains and species that are to be conserved for future use.

Genetic Inactivation of spermatozoa and eggs by irradiation in some species of fishes

Species	Dose	Reference
Spermatozoa		
O. niloticus	UV, 300-310 μW/cm^2	Hussain *et al.*, 1993
Rainbow trout	^{60}Co-gamma ray, 1kGy	Chourrout, 1984
Gold fish	X-ray, 0.75 kGy	Stanley & Sneed, 1974
Common carp	UV, 254 nm, 20 min, 10 cm apart	Jorm *et al.*, 1992
Ova		
Common carp	UV, 175 mJ cm^{-2}	Bongers *et al.*, 1995
Rainbow trout	^{60}Co-gamma ray, 3.6kGy	Parsons & Thorgaard, 1985
Brook trout	^{60}Co-gamma ray,0.88 kGy	May et al., 1988
Common carp	UV (254nm) 3-6 min; 5 cm apart	Ponniah *et al.*, 1997

Protocol for diploidization in some species of fishes

Species	Method (shock/duration/time after fertilization)	Reference
A. Meiotic gynogenesis: Retention of second polar body		
Zebrafish	560 Kg-cm^2 /5.5 min/ 1.5min	Streisinger *et al.*, 1981
Labeo rohita	12^{0}C/10 min/7min	John *et al.*, 1984
L. rohita	42^{0}C/1-2 min/ 7 min	Reddy *et al.*, 1990
Catla catla	39°C/1 min/4 min	John *et al.*, 1984
O.mossambicus	42^{0}C/3 min/2.5-4.5 min	Pandian, 1993
O.niloticus	8000 psi/2-5 min/2.5 min	Hussain *et al.*, 1993
I. punctatus	8000psi/3min/5 min	Goudie *et al.*, 1995a
Cyprinus carpio	40^{0}C/1.5 min/5 min	John *et al.*, 1992
B. Mitotic gynogenesis: Prevention of first cleavage		
O.niloticus	41^0 C/3.5 min/30min	Hussain *et al.*, 1993
O.niloticus	9000 psi/2 min/40-50min	Hussain *et al.*, 1993
Zebrafish	41.4^{0}C/2min/13min	Streisinger *et al.*, 1981
I. Punctatus	8000 psi/3 min/90 min	Goudie *et al.*, 1995
Cyprinus carpio	40^{0}C/2 min/30-40 min	**Error! Not a valid link.**
C. Androgenesis		
Common carp	40^0 C/2 min/28min	Bongers *et al.*, 1995
Rainbow trout	630 Kg-cm^{-2}/1-3 min/5.75 h	Parsons & Thorgaard, 1985
Brook trout	595 Kg-cm^{-2}/3 min/7.5 h	May *et al.*, 1988
Cyprinus carpio	42^0 C/90 sec/15-20min	Ponniah *et al.*, 1997

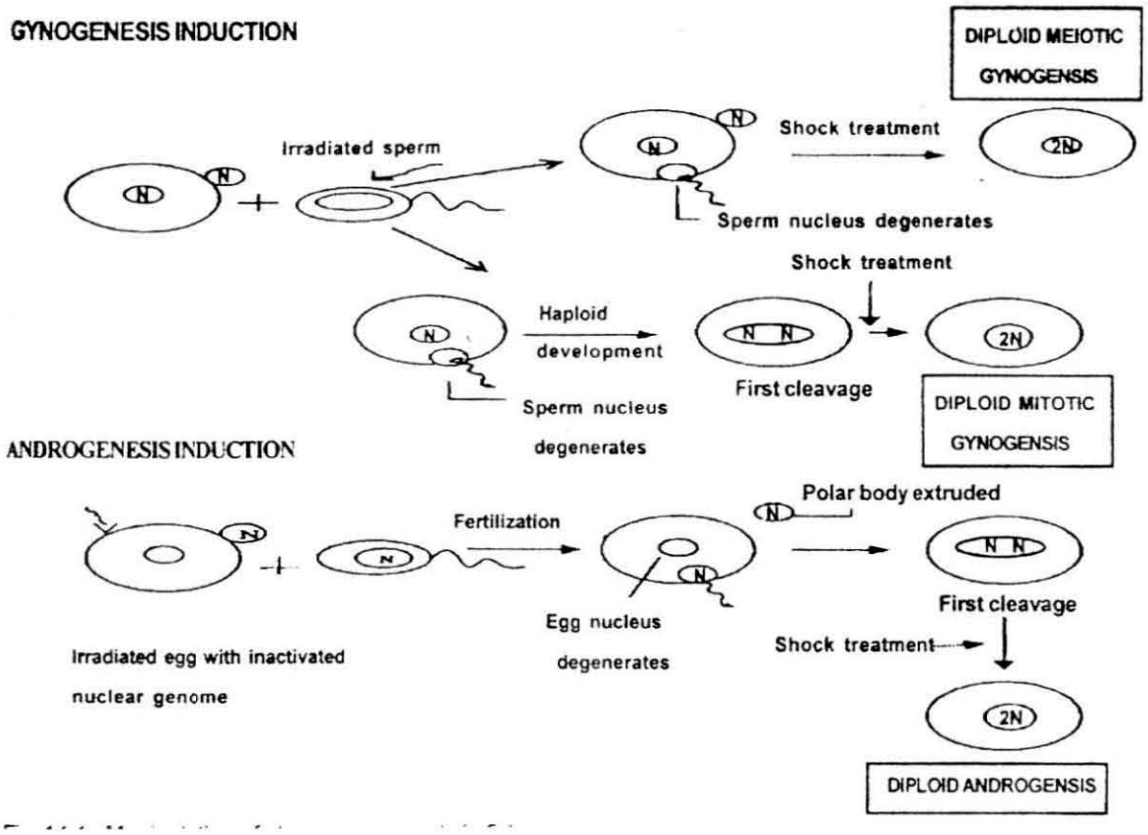

Fig. Manipulation of chromosomes sets in fish

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PLOIDY MANIPULATION

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Introduction

Each species of microorganisms, plants and animals is characterized by a particular chromosome complement or set of **genome**, represented once in gametic haploid cell and twice in somatic diploid cells. Possession of such sets of chromosomes or genomes gives to each species a specific chromosome number. But, sometimes some irregularities may occur during mitosis, meiosis or fertilization and may produce cells with variant chromosome number. Such variation in chromosome number (**ploidy**) may occur through the duplication or loss of complete chromosome sets or genomes (**euploidy**) or of single or few chromosomes (**aneuploidy**). In fishes, aneuploidy has no significance, hence only euploidy discussed below.

Euploidy: The term euploidy designates genomes containing whole sets of chromosomes. The euploids are those organisms, which contain balanced set or sets of chromosomes or genomes in any number, in their body cells. The euploidy is of following types:

1. **Monoploidy**: In monoploidy, the monoploid organisms have one-genom (n) in their body cells. When monoploidy occurs in gametes (sperms and eggs) it is termed as haploidy. Most micro-organisms (*e.g.,* bacteria, fungi and algae); gametophytic generation of plants (*e.g.,* bryophytes and other plants); sporophytic generation of some higher angiospermic plants (*e.g., Sorghum, Triticum, Hordeum, Datura,* etc.) and certain hymenopteran male insects (*e.g.,* wasps, bees, etc.) have one genome in their body cells, hence are monoploids. They are usually smaller and less vigorous than their diploid prototypes. Characteristically, monoploid plants are sterile. The reason of sterility is that the chromosomes have no regular pairing partners (homologous chromosomes) during meiosis and meiotic products are deficient in one or more chromosomes. For instance a haploid in maize (2n-20) will have 10 chromosomes in a gamete can range from 0 - 10. Consequently, considerable sterility will be found in monoploid maize.

2. **Diploidy** : The diploidy is characterized by two genomes (2n) in each somatic cell of the diploid organisms. Most animals and plants are diploids. The diploidy is related with fertility, balanced growth, great vigorsity, adaptability and survivability of the diploid organisms.

3. **Polyploidy**: The organisms with more than two genomes are called polyploids. Among plants and animals, the polyploidy occurs in a multiple series of 3,4,5,6,7,8, etc., of the basic chromosomes or genome number and thus causing **triploidy**, **tetraploidy**, **pentaploidy**, **hexaploidy**, **heptaploidy**, **octaploidy**, respectively.

Ploidy levels higher than tetraploid are not commonly encountered in natural populations, but our most important crops and ornamental flowers are polyploid, e.g., wheat (hexaploid, 6n), strawberries (octaploid, 8n), may commercial fruit and ornamental plants, liver cells of man, etc. Polyploidy is rare in animals, however has been reported in *Ascaris, Daphina, Cypris, Drosophila, Bombyx mori, Hbrobracon,* bees,wasps, *Artemia, Echinus, Rana esculenta, Rana pipiens, Ambystoms, Jeffersoniarium, Cricetus cricetus* (hamaster) *etc.*

Polyploidy occurs naturally in some groups of fishes, like *Misgurnus anguillicaudatus etc.* and it can be induced artificially. Mahseers, Common carp, Gold fish and some mullets are also considered as natural tetraploids. First extensive studies on artificially produced triploid fish was done on the [illegible] by Swarup (19[illegible]). He reared [illegible] and fertility with diploids. Mass production of tetraploids was achieved for the first time in Rainbow trout by using pressure shock. To produce polyploid seed on a commercial scale standard protocol for the ploidy induction and efficient screening method for their identification is necessary. Amenability of some fish species (*M. anguillicaudatus*) to chromosome multiplication is amazing! Experimental penta, hexa and heptaploids only in Japanese loach *Misgurnus anguillicaudatus*; and rainbow trout (*O.mykiss*).

Kinds of polyploidy: Following two main kinds of polyploidy, auto and allopolyploidies have been distinguished on the basis of the source of chromosomes. (1) **Autopolyploidy** - The prefix "auto" indicates that the ploidy involves only homologous chromosome set and (2) **Allopolyploidy** - The prefix "allo" indicates that non-homologous sets of chromosomes are involved. The union of unreduced or diploid or polyploid gametes from different diploid or polyploid species could produce in one step, an amphipolyploid or allopolyploid, which appears and behaves like a new species.

Chromosome Manipulation in fish

Chromosome manipulation in fishes is useful in population control, growth improvement and production of inbred lines. Chromosome sets can be manipulated by meticulous application of temperature (cold and heat), pressure or chemical shock. Shock treatment disrupts the normal cycles of mitosis and meiosis. It suppresses the extrusion of second polar body or arrests the first cleavage division. This is necessary for diploidizing gynogenetic and androgenetic offspring and in inducing triploidy and tetraploidy.

1. **Shock treatment** : (a) **Thermal shock**: Cold shocks are usually applied near 0^0C for cold water species such as salmonids and somewhat at higher temperature (8-12 ^{0}C) for warm water species like common carp, Tilapia and Indian carps. Heat shock is applied at a lower temperature (around 26-28^{0}C) in the case of cold water than in warm water fishes (39-42^{0}C). (b) **Pressure shock**: This method is simple to administer. The pressure range varies between 7000 to 9000 pascals (Psi). The hydrostatic pressure is applied by a specialized instrument designed by mechanical engineering method. Pressure shock is supposed to have less side effect than the

thermal shock. But given in a sub-optimal intensity, pressure shock produces aneuploid offspring and (c) **Chemical shock**: Colchicine and cytochalasin-B have the potential to disrupt cell division and induce ploidy induction. But the results are inconsistent and unsatisfactory. Anesthetics such as nitrous oxide and Freon 22 have also been tried to induce triploidy.

The duration and dose of thermal or pressure shock and the optimum conditions have to be found out by trial and error process. Table 1. Summarizes the conditions applied for manipulating the chromosome sets in some species of fishes.

Protocol for induction ploidy in some fishes

Species	Method (shock/duration/time after fertilization)	Reference
A. Triploidy: Retention of second polar body		
Common carp	40 or 41°C/2 or 1.5 min/6 min	Recoubratsky *et al.*, 1992
Grass carp	5-7°C/25-30 min/2.0 - 4.5 min	Cassani & Caton, 1985
Laboe rohita	42°C/1-2 min/ 7 min	Reddy *et al.*, 1990
O.mossambicus	42°C/3 min/2.5-4.5 min	Varadaraj & Pandian, 1990
Ictalrus punctatus	7500 psi/2-5min/2.5 min 0°C/1 hr/5min	Wolters *et al.*, 1981
Heteropneustes fossillis	4°C/30 min/ 2min	Tiwary *et al.*, 1997
B Tetraploidy: Prevention of first cleavage		
Rainbow trout	490 Kg-cm^2 4 min/ 5.8 hr	Chourrout, 1984
O.aureus	11.0°C/ 60 min /80-104 min	Don & Avtalion, 1988
I. Punctatus	40°C/80-90 min/ 3 min	Bidwell *et al.*, 1985
Plecoglossus altivelis	650 kg-cm^{-2}/ 6 min/80 min	Taniguchi *et al.*, 1990

Detection of polyploid individuals

The production frequency of polyploidy individuals varies if the shock treatment is not given in proper dosage at appropriate time. It may so happen that a mixture of [illegible] The diploid and [illegible] morphology but can be distinguished by following methods:

i) *Chromosome analysis* is the simplest and appropriate method for determining the ploidy level. But it is very much labour intensive and time-consuming.

ii) *By the measurement and comparison of the nuclear volume and cell volume of the erythrocytes*, the ploidy level can be distinguished. This can be done with the help of light microscopy. The electronic instruments like Coulter counter can be used to measure the cell size rapidly.

iii) *Flow cytometer* is an instrument that helps to detremine the DNA content and cell size. Flow cytometry involves staining the cells with a DNA specific fluorescent dye, propdium iodide, followed by quantification of fluorescence upon laser excitation.

iv) *Counting of the number of nucleoli* after silver staining method that can be applied in those species having a constant number of nuclear organizer regions (NORs) per cell. However, it is not a suitable methods if the number of NOR varies per cell.

v) *Isozyme analysis* can be applied insome cases. For example, electrophoretic examination of Phospho Gluco Isomerase (PGI). Esterase and other allozymes in Brown trout (*Salmo trutta fario)* and in some Cyprinids could distinguish the diploid and [illegible] individ[illegible]

Evaluation of polyploid fishes

a) **Survival**: Triploid fishes survived better than the tetraploid ones. The survival of autotriploid was lower than the allotriploids. Triploid hybrids survived in a greater percentage than the diploid hybrids in the crosses between Rainbow trout, Brook trout and Brown trout. Triploid hybrids of Chinook salmon and Pink salmon also exhibited greater survival than the diploid hybrids at the juvenile stage. The survival of triploid Common carp was, however, lower than the diploid controls.

b) **Growth** : The effects if triploidy on growth rate differ in different species. The reason for such variation is not fully understood. The growth rates were similar in triploid and diploid fishes of poecilopsis, stickleback, coho salmon, *Clarias gariepinus* and *Oreochromis niloticus.* Triploid coho salmon grew slower than the diploids in juvenile stages. In case of Channel catfish, diploids grew slower than the triploids after attaining maturity. Triploid *Clarias macrocephalus* showed higher survival rate, increased body weight and low rate of deformities.

c) **Reproduction**: induced polyploidy affect fertility in females more than in males. In triploids, the gonadal development is generally lesser in females than in males. In diploid and triploid *Orechromis mossambicus* , the testes developed to the same size but the ovaries of the triploids remained markedly under developed. The triploid males of Chinook salmon and Rainbow trout developed sexual phenotypes but produced aneuploid sperm, which had the capacity of forming viable offspring. In contrast, all triploid females failed to undergo ovarian development and lacked sufficient levels of sex steroid necessary for the maturation process. Ovary was very much reduced in triploid Grass carp female. In Channel catfish, Plaice and Plaice x Flounder hybrid, the ovaries in diploid individuals were four times larger than the triploids.

Despite well-developed testes in triploid Channel catfish, Atlantic salmon and laoch, matured spermatozoa were not produced. Testicular growth and spermatogenesis were nearly normal in triploid Grass carp but abnormal aneuploid spermatozoa were produced. Triploid common carp was found to be sterile in both sexes. Triploid females of *O. mossambicus* were found to be sterile and showed

increased rate of stomatic growth. These females grew 14% faster than the monosex (sex reversed) males and 23% faster than the triploid males.

Tetraploid male and female Rainbow trout produced milt at the age of two years and it could be crossed with the diploid females to produce viable triploid offspring. The diploid sperm of tetraploid males are larger and may face difficulty in penetrating the micropyle of normal (haploid) eggs. Further, the tetraploids produce aneuploid gametes due to improper separation of four homologous chromosomes during meiosis. Survival rates of tetraploids are very less due to mosaicism, wrong cell division events, high homozygosity and decreased cell number & increased cell volume leading to reduced cell surface.

Applications of polyploidy in aquaculture

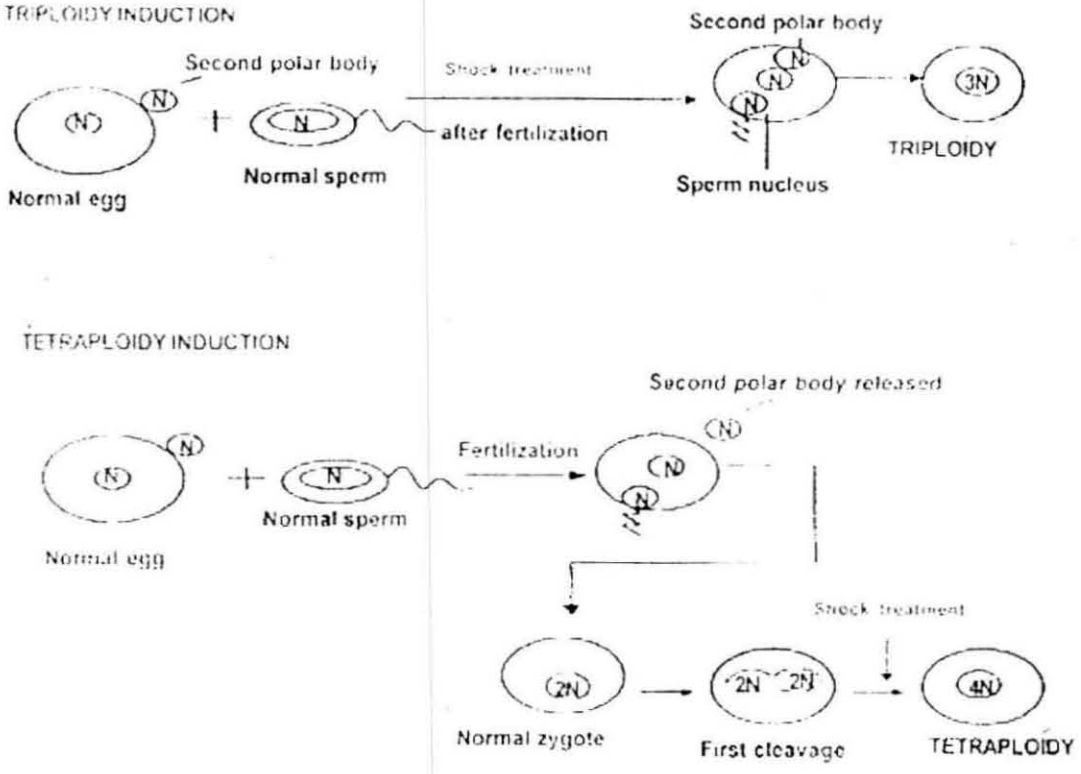
Polyploidization may enable an animal to adapt to varying environmental conditions. Common carp and the catostomid fishes are naturally polyploid species. It is believed that the polyploid genome enables these species to live in diverse ecological conditions. Triploid hybrids are more viable than diploid hybrids. Triploids are also more heterozygous and heterozygosity helps in maintaining their developmental stability.

Triploidy led to sterility in some fishes like Grass carp and Channel catfish. Triploid Grass carp cannot breed in natural waters and thus does not pose any environmental problem if transplants into larger water bodies for weed control. The culture of triploid Channel catfish is more profitable.

Sterile triploid progeny can be produced by crossing tetraploids with diploid ones. Triploid Rainbow trout produced by mating of tetraploid female with diploid male exhibited higher growth rate and survival than artificial triploids. Diploid spermatozoa of tetraploid males are useful for producing androgenetic offspring.

In India research on induction of triploidy is mainly undertaken at the Central Institute of Freshwater Aquaculture (CIFA), Bhubaneshwar, where triploids of most of the major carps and their hybrids have been produced. In the Central Marine Fisheries Research Institute (CMFRI), Cochin, the technology has been perfected for the release of both first and second polar bodies by chemical (6-DMAP) treatment by a team led by Dr. P.C. Thomas. They observed 30% increase in growth and 2 fold increase in glycogen and lipid content in triploid oysters.

Fig. Production of triploid and tetraploidy in fish



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TRANSGENIC FISH

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Definition

Transgenic animal is defined as an animal that has been become transformed following the introduction of a novel gene in to its DNA. Gordon et al (1980) were the first to produce transgenic animals leased on the microinjection of cloned DNA into pronucleus of the fertilized eggs at the arc cell stage. Palmitev et al (1982) INTRODUCED GH gene into mice and achieved dramatic increase in growth of the animals and the successful transgenic animal has been produced. Thus a 'giant' mouse was produced – normal 29gms- the giant mouse grew up to 44gms. Sadly the giant mouse died prematurely.

However, Introduction of novel genes and production of transgenic fishes were very slow- it was only in (1985) Zhu et al introduced novel genes into gold fish (CARASSIUS AURATUS L 1758). The gene introduced was growth hormone gene. Gill et al (1985) showed that chicken growth hormone, and bovine growth hormone accelerated growth in Pacific salmon. Soon fish growth hormone gene was isolated and expressed. Now many laboratories are working in this area.

2. Advantages of fish as transgenic animals:

i). Large number eggs are available.
ii). Can be fertilized under controlled conditions.
iii) Need not to return to the female reproductive tract for completion of development as in the case of mammals.
iv) Availability of a large number cloned (c DNA) genes.
v) Transgenic fishes are recognized as the superior system for examining the molecular development of early vertebrate development.
vi) Transgenic fish may play an important role in aquaculture.
vii) There are no ethical considerations, which restrain gene manipulation on higher animals.
viii) Transparency of embryos in many species.
ix) Haploid, Polyploid individuals are available.
x) Gynogenetic and androgenetic individuals are available to study the system.

3. REQUIREMENT FOR PRODUCTION OF TRANSGENIC FISHES:

Three steps are involved in the production of transgenic fishes

a) Specific recombinant DNA construct must be prepared having the gene of interest or the regulatory element in it.

b) The recombinant DNA must be delivered to the nucleus of the cell of the developing embryo/ fertilized egg in order to be distributed to all the tissues of the fish.
c) Since all deliveries of the trangency will not be effective and not all constructs will behave in the manner that was desired, a screening process must be established.

3.1 .**VECTORS**

For producing transgenic fishes the genes should be cloned in to the expression vectors. Expression vectors have regulatory sequences that determine where, when and the level at which the transgene will be expressed. All the vectors so far used in transgenic fish studies show the following general factors. They are all plasmid vectors can be replicated to the level of 500 to 2000 copies per cell with selectable and unique restriction sites.

6.6Kb Bam H1 and EcoR1 fragment used for microinjection.

This plasmid contains the mouse metallothionine gene and rat growth hormone gene having the necessary regulatory elements and gave good expression. The use of heterologus GH genes was found to be a problem, such as improper splicing and translation. Further the use of heterologous genes was mainly due to the lack of availability of suitable piscine sequences. Many workers have developed all fish vector – Liu and coworkers used Carp beta actin gene enhancer –promoter with Ploy (A) addition signal from salmon.
These vectors expressed in all tissues. Another group has (Hew et al)
constructed all fish expression vectors using antifreeze protein gene promoters-This vector did not express in all the tissues. Since beta actin promoters are stronger than AFP promoters constructs with trout metallothionine gene are also available but they are not preferred because heavy metals such as Zinc are to be used for inducing the transcription of the genes.

The vector should contain introns, enhancers, boundary regions, control regions in addition to promoters for the proper expression (Palmitiv et al 1991).

3.2 REPORTER GENES:

There must be suitable reporter genes to see the presence of the transgene in the various tissues of the animals. Reporter gene is defined as a gene whose products detects or marks the cells, tissues, organisms that express the gene from those that do not. Reporter genes isolated from prokaryotes, E.coli, are used in fishes – Lac Zgene , Cat(Chloramphenicol Acetyl transferase gene). Recently luciferase and green fluorescent protein are used.

3.2.1. **LUCIFERASE:**

This is the most sensitive and one of the simplest reporter systems adopted in transgenic fish. This gene is isolated from firefly PHOTINUS PYRALIS. Previously they used to cell/tissue homogenates where the reaction was started by the addition of luciferin substrate containing Acetyl CoA and ATP. The enzyme activity was monitored by photon emission by scintillation counter.

In 1990 TAMIYA et al demonstrated for the first time that the incubation of living MEDAKA embryos in a solution of luciferin resulted in the penetration of the substrate in to the living tissues thus allowing the detection of the expressing embryos by using a photon counter or by if the embryos are placed on a very sensitive X-ray film they can be detected.

Advantages:

It has many advantages over other reporter systems.

a) Luciferase is a simple polypeptide, which does not require post translational modification.
b) There is no endogenous background of any sort.
c) Shows linear activity upto 10^6 units.
d) The half-life is only 3 hours.
e) It is also a quantitative assay if tissue homogenate are used.

3.2.2 **GREEN FLOURESCENT PROTEIN:**

This a novel gene isolated from a jellyfish AEQUOREA VICTORIA. Light is produced when calcium binds to this protein. GFP has 238 amino acids absorbs blue light at 395 nm and emits green light at 509 nm. This fluorescence is stable. Use of this as a reporter gene has many advantages.

a) It does not require external substrate over it is to be irradiated by blue light. So not limited by availability of the substrate.
b) So gene expression can be monitored in living cells / animals.
c) The substance is not toxic to living cells/ animals.
d) It is more useful in embryos, which are transparent as ZEBRA fish embryos.
e) Since this protein is persistent even in formaldehyde fixed tissues it can be examined later also.

3.3 **GENES OF INTEREST:**

GROWTH HORMONE GENE

It is a circulatory hormone, which induces growth. It also facilitates seawater adaptation and stimulates gonadal steroidogenesis in salmon. Experiments have shown that transgenic pacific salmon was 11 times heavier than control (highest was 37 times heavier than control). However, There was an increased mortality in transgenics.

ANTIFREEZE PROTEINS

This protein was found in fish inhabiting cold water. They lower the freezing point of the plasma. They are largely synthesized in liver and released into the circulation.They are classified into three types depending on the amino acid sequences. Attempts are being made to increase tolerance of salmon, which lack AFP, and this can allow them to be cultured in north Atlantic waters. However AFP can give general cold tolerance.

DISEASE RESISTANCE

Fish has poorly developed antibody dependent immunity. The antibacterial enzyme lysozyme found in blood, mucous, kidney and lymphoid tissues play a role in nonspecific line of defence. Rainbow trout lysozyme if potent inhibitor of gram negative bacteria such as Vibrio anguillarum, V.harveyi and Aeromonas etc.

METALLOTHIONINE (MT):

It is a regulatory protein. It can be induced by $ZnCl_2$. This can be used for monitoring pollution of water. They're also other genes that can be used for developing transgenic fish.

3.4. **METHODS OF GENE TRANSFER:**

For production of transgenic animals the gene should be transferred successfully. There are many ways by which the genes can be delivered to the cells. The most important are Microinjection, Electroporation, Sperm delivery, Lipofection, Particle bombardment (Gene guns)

3.4.1: **MICROINJECTION**

This technique was originally was developed for mouse eggs and is used in fishes also. This is very efficient in mouse because of smaller number of eggs and small size of egg and the pronucleus are visible and the eggs are not surrounded by hard shells. Fish eggs are large 1-7 mm in diameter, (300 to 30,000 times larger than mammalian eggs). Cellular / nuclear volume ratio in mammalian egg is 20 whereas in fish egg is >100,000.Hence seeing the pronucleus is very difficult. Further the rapid onset of cleavage and hardening of the chorion. In spite of the difficulties this procedure is straight forward and attempts are made to adopt this procedure to suit fish eggs. Some species has softer chorion such as Catfish, Zebra fish. Small volume of the solution 1-2 nl of DNA containing $>10^7$ copies should be injected. The survival rate after injection is 80-80%. High level of mosaicism (90-99%) of the integration is noticed suggesting that the integration has not occurred efficiently. Further the first cell division is after 30 minutes after fertilization. So it is difficult to handle large number of eggs. Hence there is an advice for developing efficient procedures for mass transfer.

3.4.2: **ELECTROPORATION**

Electroporation is a technique based on exposing the cell membranes to high intensity of electric field pulses. As a result specific regions of the cell membranes are temporarily destabilized. During the stabilization the cell membrane is highly permeable to exogenous molecules present in the surrounding media. This method has been successful for gene transfer to prokaryotes and eukaryotic cells in culture. Since 1990 it is gaining favour among research workers. INOVE and his coworkers demonstrated that electroporation of medaka+ eggs resulted in 25% survival and 4% of those had transgenic fishes (800ml of solution having 80mg DNA)They used 100ng DNA /embryo capacitor driven 50milli seconds pulses at 750V/cm. In Zebra fish they used 0.1milli second pulses of 125V/cm for batches of 200 eggs. However each experiment has to be standardized as per the requirement to obtain optimum success.

3.4.3: PARTICLE BOMBARDMENT (GENE GUNS)

High velocity micro projectiles have successfully used to permeate the thick sell walls of certain fish eggs to deliver the transgenes. Primarily it was used in plants to transform chloroplasts. A thin coat of DNA is coated on to the surface of the 1µm diameter tungsten or gold particles by precipitation with calcium chloride. The beads are placed on the end of the plastic bullet (macro projectile) in the barrel of the gun specially designed for this purpose. The eggs are placed in suspension at the end of the barrel. The particles are sent towards the eggs by high speed and hits the cells. The barrel of the gun and the specimen chamber have to be evacuated, otherwise the speed of the particles are reduced. This technique was used for Zebra fish, Trout 75% survival and 5% transgenics were found. This is also a promising technique.

3.4.4: **SPERM CARRIERS**

Lavitrano showed that the mouse sperm could be used to convey externally cloned transgenic DNA into recipient eggs. Many fish workers have attempted to use this technique. This technique works in fish also. However, there was no evidence of chromosomal incorporation or successful F1 progeny. A modified procedure was developed ie sperm were electroporated, so that transgenes were internally confined to the sperm and carried on to the eggs with out injury. This also works in some case but not successful

3.4.5: **LIPOFECTION**

Here synthetic lipid vesicles encapsulate nucleic acid or nucleic acid protein complex and permit their uptake in to cells following fusion of the vesicles with the plasma membrane. Dechorionated catfish eggs were subjected to lipofection. Depending on the liposome/embryo ratio transformation frequencies were obtained resulting in the transgenic animals. Success ranged from 60 to 80%.

All the methods show clearly that there are two sides for expression. One is delivery and the second is expression. For successful production integration of the transgene must take place such that it is stable.

4. **DETECTION OF TRANSGENE**

After the introduction of the transgene the next question is to find out whether the gene has gone on to the system. There are various ways and means to detect this. Most of them used southern blot and northern blot to detect the transgenes using a suitable probe. (Restriction digest, gel electrophoresis, Filter transfer, labeled nucleic acids and probing) Then northern blot (dot blot) shows whether the DNA is synthesizing the mRNA and expressed. Western blot – see whether the desired protein is synthesized. PCR-through PCR also it is possible to detect small tissue like fin or other–template DNA then primer PCR.

5: **FATE OF THE INJECTED DNA:**

The transgene may be present in the animal but it may exist in various forms. It may not be present in the form, which is required for permanent transmission from one generation to another.

5.1: **DELIVERY OF DNA**

Transgenic DNA is generally delivered

a) super coiled plasmids containing both vector and transgenic DNA.
b) Linear DNA without vector DNA.
c) Concatemeric DNA of the transgenic DNA.

In general transgenic animals will not have bacterial vector. Therefore only transgenic DNA is injected. The linear DNA was better incorporated compared to circular DNA. Further the regulatory elements of prokaryotes are not essential for the fish. Further transgenic requirement emphasis that transgene material should not be evolutionarily distant (They should be close)

5.2: **MOST OF THE DNA IS DEGRADED**

Since cytoplasm is rich in exo and endonucleases most of the DNA degraded. However the digestion is not immediate.

5.3: **SOME COPIES OF THE DNA MAY PERSIST FOR LONG TIME BUT WITHOUT INTEGRATION:**

It has been shown that the DNA is replicated and undergoes conformational changes and only after many days the DNA disappeared. The DNA persist for a long time and get distributed to many tissues, such genes form concatamers.

5.4: **INTEGRATION AFTER PARTIAL DEGRADATION:**

Since degradative enzymes act on the DNA it is possible that a portion of the sequence may be lost before integration.

5.5: **INTEGRATION OF ONE COMPLETE COPY OR MANY COPIES:**

There is also a possibility that the whole of the DNA is integrated. However the precise location of the integration may not be known. Sometimes they may persist as pseudochromosome where centromere is in the transgene.The integration may be at many sites or at one site as concatamer.

5.6: **INTEGRATION AFTER SOME ROUNDS OF CELLDIVISION:**

This will lead to production of mosaics either because they are integrated in different sites at different tissues or integrated in some tissues and not in others. The mosaicism will lead to lack of integration into germ line cells and hence cannot be transferred to germ cells.

5.7: **INTEGRATIONWITHOUT EXPRESSION:**

Normally the integrated genes are expressed. Due to inactivation through DNA mehtylation or lack of satisfactory promoters or integration closed to heterochromatin the gene s may not expressed even though integrated.

5.8: **APPROPRIATE EXPRESSIONS:**

The desired result is the appropriate expression. In some cases it must tissue specific but in some cases it need not be. This will result in the phenotypic expression. This fact can be assayed by protein, or immunology or RNA blots.

5.9: **INAPPROPRIATE EXPRESSION:**

Expression will be appropriate if they are expressed in correct tissue and time.

5.10: **GERMLINE TRANSMISSION:**

The final goal of most transgenic work is the germ line transmission many worker were able to achieve this.

5.11: **CONCATAMERISATION:**

This is found just after infection of DNA of both in linear and circular. This may be [illegible] large amounts of DNA infection may result in this.

5.12: **REPLICATION:**

In fishes it is often found that the infected DNA, irrespective of the DNA sequence replicate in an extrachrosomal way. This correlates with the rapid DNA synthesis that takes place in the developing embryo.

5.13: POSITIVE EFFECTS:

The position of integration of the transgene in the chromosome shows influence /effect on the expression of the gene –called position effect. This accounts for the copy number and the level of expression. This is very obvious in transgene, which not does not

contain strong promoters/silencers. The inclusion of introns in the transgene has been found to enhance the expression and laso copy number dependent expression. Certain DNA sequences such as present in the boundaries of chicken lysozyme gene, hsp 70 genes when attached to the transgene the expression are more uniform.

5.13: **DNA METHYLATION:**

Transcription of RNA ploymerase II genes can be inhibited by DNA methylation. Novel genes upon integration become methylated and consequently inactivated. The mechanism is not clear. However, integration disrupts the normal chromosomal structure. If the embryos are treated with methylation inhibitor (5-azacytidine) better expression is found.

6: **FUTURE OF TRANSGENIC FISH:**

What is the outcome of the exercise?

6.1: **GROWTH ENHANCEMENT:**

This was one of the advantages **of** producing transgenic fish. Pacific salmon showed a dramatic growth increase. There are other fishes, which show enhanced growth such as carp, trout, and catfish. The aspects of growth

a) Initial fast growth so that they attain sexual maturity earlier.
b) Enhanced growth as adults –good for marketing.
c) Improved feed efficiency.

When we use Promoter such as viral or meatllothionine they are not accepted by the public.

6.2: **COLD TOLERENCE:**

In western countries and In our countries places like Himalayas and hill region people are interested in growing fish in cold areas. So fish should be able to withstand cold. So AFP genes are injected they may be able to withstand cold and grow better.

6.3: **PRODUCTION OF IMPORTANT PHARMACEUTICAL PROTEIN IN FISH:**

Use of fish as a bioreactor.

6.4: **IMPROVED DISEASE RESISTANCE:**

Fish are prone to viral and other diseases. Antisense RNA or Lysozyme genes it would be possible to produce fish, which show some resistance to diseases.

6.5: **STUDY OF VERTEBRATE DEVELOPMENT:**

Fish owing to its easy handling, short generation etc can be used as a model organism to study vertebrate development. To study enhancer/promoter / silence activity genes from other organism, mutageneis etc.

FURTHER APPLICATION OF TRANSGENIC FISH:

a) Better understanding of development, growth, gene regulation in fish and the reproduction process.

b) Improved economics of fish culture.
    - Improved feed conversion efficiency.
    - Improved cold resistance.
    - Improved freeze resistance.
    - Improved disease resistance.
    - Improved fecundity of brood stock.
    - Utilize low cost feed
c) Fish as bioreactors.
    - Production of medically important compounds.
    - Production of commercially important non-medical compounds.
d) Production of tailored fish.
    - With improved flavour.
    - With improved color, texture etc
    - With fatty acid composition.

Fish, as a transgenic animal will contribute more to the service and economics than any other organism because of its suitability and easy to handle.

GENE CLONING – TECHNIQUES AND STRATEGIES

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Introduction

Genetic information is coded in DNA found in the nucleus and mitochondria of animal cells and chloroplast of plant cells. The flow of genetic information from DNA to RNA and subsequently to polypeptides or proteins forms the central dogma of molecular biology. From the complex pool of DNA how can one isolate and study a specific sequence, or in other words, a gene of interest? The technology of gene cloning allows the amplification and recovery of a specific fragment from the highly complex DNA pool so that it can be comprehensively studied with regard to its structure, function, regulation, expression, etc. This technology has been immensely useful in the areas of medicine, agriculture and industry. For example, it is now possible to produce proteins to be used as diagnostics and therapeutic agents through recombinant DNA technology. The term must be distinguished from the natural DNA recombinants that result from crossing over between homologous chromosomes in eukaryotes and prokaryotes. It is used in the sense of unnatural union of DNAs from non- homologous sources, usually from different organisms. The alternative term chimeric DNA is derived from the Greek mythological monster Chimera, a combination of parts of different animals.

In practice, DNA cloning can be achieved in two different ways: cell based cloning and PCR based cloning.

Cell based cloning

Principle and approaches

Cell-based DNA cloning strategy is the most popular method in which the novel combination of DNA from a 'donor' organism and a 'host' organism would take place through a 'vector'. The basic procedure involves the following steps:

- Extraction of DNA from the donor organism ('foreign DNA') and vector (such as plasmids, bacteriophages, etc.)
- Cutting it up into several fragments using restriction endonucleases
- Inserting the cut donor DNA into cut vectors
- Introducing the recombinant DNA into host cell (e.g. *E. coli*) and propagation of host cells in suitable culture medium.

Several clones of host cells are grown in the medium containing several fragments of the donor DNA and it will be the most important job to select the clone containing the gene of interest. Figure 1 gives a brief out line of procedure.

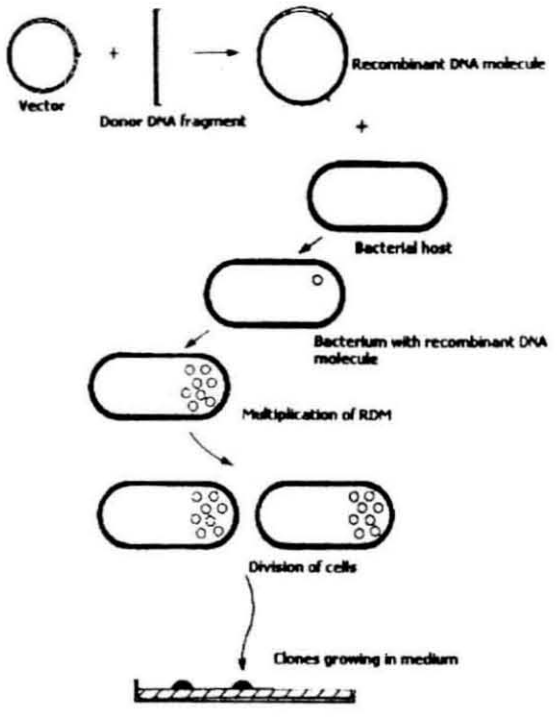

Fig. 1 The basic steps involved in gene cloning

Extraction of DNA from the donor organism ('foreign DNA'): Genomic DNA from the donor would be extracted using standard protocols, the most common being the proteinase K digestion followed by phenol-chloroform extraction (Sambrook *et al.*, 1988). Vector, such as plasmid carrying genes for resistance to antibiotics can be separated from the bacterial chromosomal DNA. Because differential binding of ethidium bromide by the two DNA species makes the circular plasmid DNA heavier than the chromosomal DNA, the plasmids form a distinct band on centrifugation in a cesium chloride gradient and can be separated.

Cutting it up into several fragments using restriction endonucleases: Restriction enzymes act as molecular scissors, cutting DNA at specific sites situated in the recognition sites. Restriction sites are not relevant to the function of the organism, nor would they be cut in vivo because most organisms do not have restriction enzymes. There are three categories of restriction endonucleases, type I, type II and type III out of which only type II find application in molecular biology. The recognition sites generally vary from 4-8 bp and are mostly palindromic in nature. This means, the recognition sequences are same on both the strands when read 5′ 3′ direction. Some enzymes make a 'staggered cut' within the recognition sequence resulting in single stranded sticky ends conducive to the formation of recombinant DNA, while cut of others result in blunt ends.

Inserting the cut fragments into a cut vector: Figure 2 shows the method of generating a chimeric DNA; the vector is a plasmid that carries one *Eco* RI restriction site and the donor molecule also has *Eco* RI sites. When the two populations of restriction enzyme-digested

fragments are pooled, they unite due to the presence of sticky ends. At this stage, although duplexes are formed to generate a population of chimeric molecules, the sugar-phosphate backbones are still incomplete at two positions at each junction. These fragments can be linked permanently by the addition of the enzyme DNA ligase, which creates phosphodiester bonds at the joined ends to make a continuous molecule.

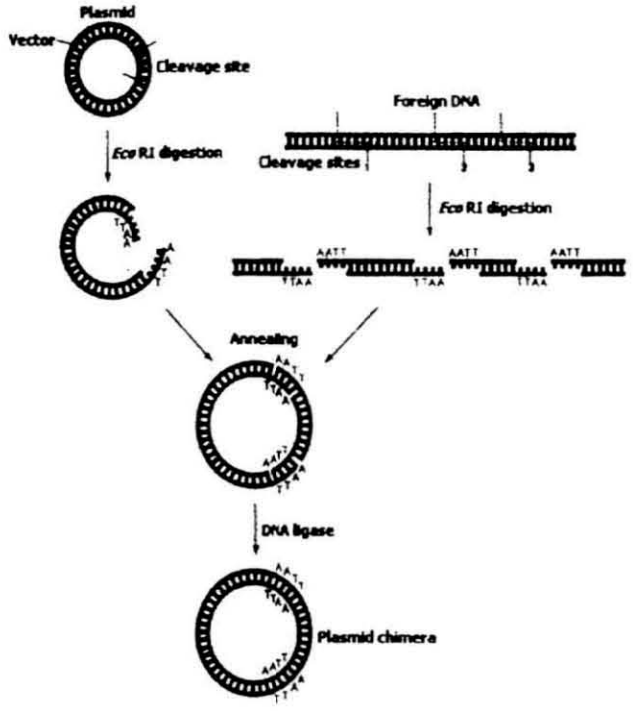

Fig. 2 Method for generating a chimeric DNA plasmid containing genes derived from foreign DNA

Introducing the recombinant DNA into host cell (e.g. E. coli) and propagation of host cells in suitable culture medium: Recombinant plasmid DNA is introduced into competent host cells by transformation. Once in the host cell, the vector will replicate in the normal way, but now that the donor DNA is a part of its length, the donor DNA is automatically replicated along with the vector. Each recombinant plasmid that enters the cell will form multiple copies of itself in that cell. Subsequently many cycles of division will occur, and the recombinant vectors will undergo more rounds of replication. The resulting colony of bacteria will contain billions of copies of the single donor DNA insert. This set of amplified copies of the single donor DNA fragment is the DNA clone (Fig. 3).

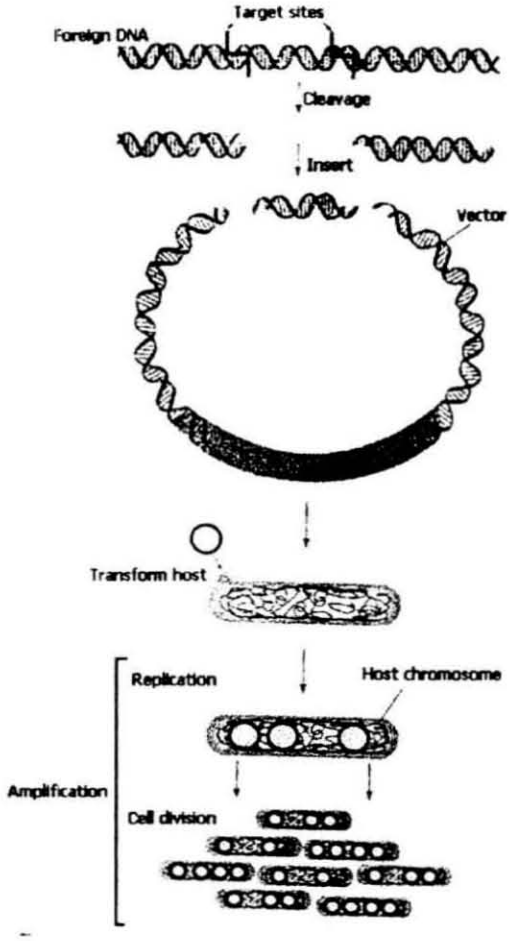

Fig. 3 Amplification of recombinant DNA in host cell

Choice of vectors

Some of the basic essential features of vectors used in recombinant DNA technique are as follows

- Must be relatively small molecules for convenience of manipulation.
- Must be capable of prolific replication in a living cell, thereby enabling the amplification of the inserted donor fragment
- Presence of multiple/unique restriction sites for insertion of DNA to be cloned

Important cloning vectors are plasmids, bacteriophage, cosmids and single stranded phages, such as M13. Some of the important expression vectors (those in which cloned gene can be transcribed and translated into proteins) include YACs (Yeast Artificial Chromosome), in which upto 1000 kb fragment can be cloned and BACs (Bacterial Artificial Chromosome), which can clone upto 300 kb fragments. Excellent descriptions of these vectors appear in reviews by Griffiths *et al* (1996) and Brown (1990).

DNA library

The specific approach to clone a gene depends to a large degree on the gene in question and on what is known about it. Generally the procedures start with obtaining a large collection of clones made from the original DNA sample. The collection of clones is known as a DNA library. This step of making a library is sometimes referred, as 'shotgun approach' because the experimenter clones a large sample of fragments hoping that one of the clones will contain a "hit" – the desired gene.

Two basic methods are available for construction of a DNA library, depending the starting DNA: *Genomic DNA library* and *cDNA library*. In case of genomic DNA library, the starting material is the genomic DNA of various sources. Typically, pure genomic DNA is cleaved by restriction endonucleases, preferably 4 bp cutters such as *Mbd* or *Sau* 3A in order to achieve fragmentation of the genome. The optimal fragmentation can be achieved through partial digestion involving low enzyme concentration and shorter incubation time. These fragments after purification and modification can be inserted into suitable vectors for cloning. Though any vector can be used for this purpose, bacteriophage λ vectors are often the choice because inserts up to 25 kb can be easily cloned in this vector (Fig. 4).

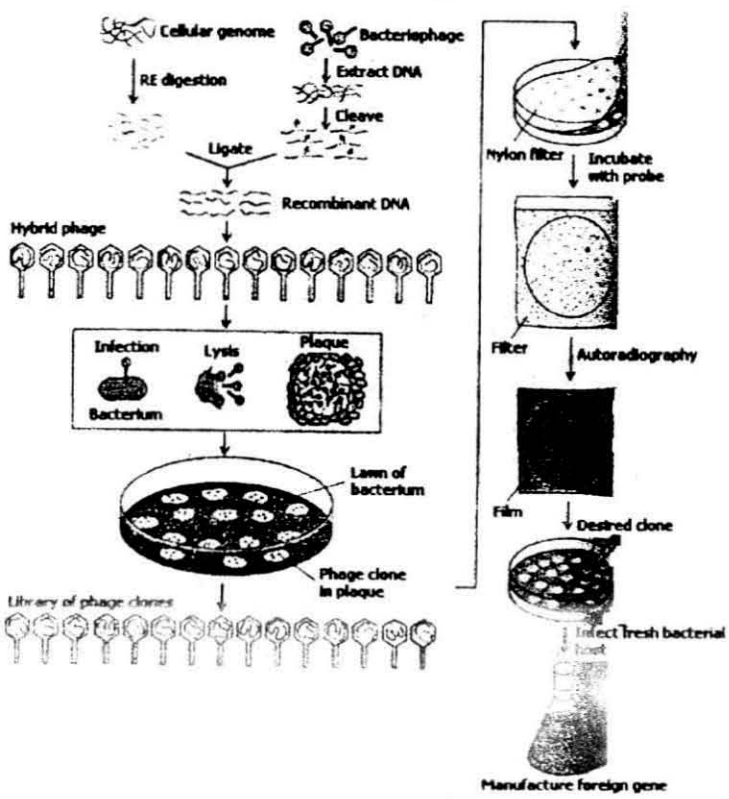

Fig. 4 Construction of a genomic DNA library from λ phage

In case of cDNA library, the starting material is usually total RNA from a specific tissue. Using the total RNA as template and enzyme reverse-transcriptase cDNAs are produced (Fig. 5). Messenger RNA too, is often used as template. The resulting double stranded cDNAs are cloned into suitable vectors.

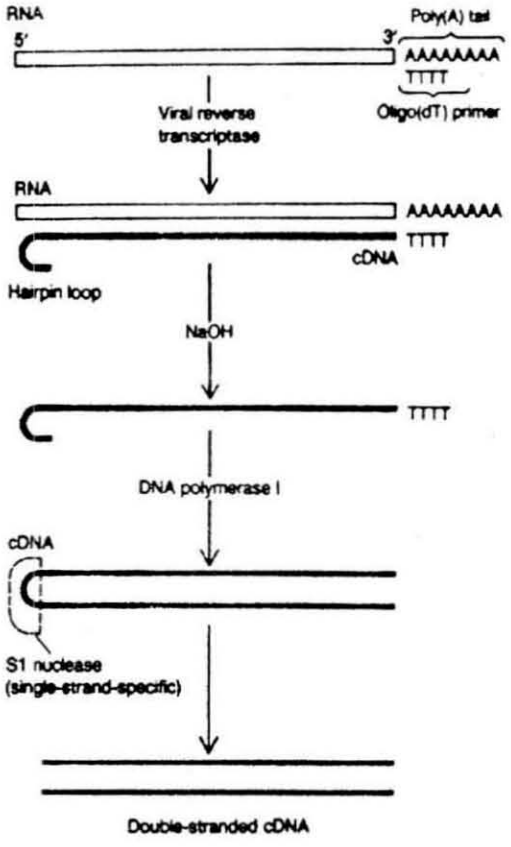

Fig. 5 Synthesis of double stranded cDNA from mRNA

The choice between genomic DNA and cDNA depends on the situation. If the gene sought were active in a specific type of tissue in the fish or shellfish, then it would make sense to use that tissue to prepare mRNA to be converted to cDNA, and then make the cDNA library. Because it is made from mRNA, the cDNA would lack in regulatory sequences and introns and thus can be translated into functional proteins in bacteria, a very important feature when cloning and manipulating eukaryotic genes in bacterial hosts. On the other hand, though the genomic library is bigger, they do have genes in native form, including introns and regulatory sequences. But the disadvantage is location of a specific gene from a genomic library is far too cumbersome.

How to find a specific clone from a library?

The library, which might contain as many as hundreds of thousands of cloned fragments, must be screened in order to find the recombinant DNA molecule containing the gene of interest. This is accomplished by using a specific *probe*. The probe comes from three sources, (a) from cDNA, (b) from a related organism, e.g., based on a gene sequence from a

related species. This depends on the evolutionary conservation of the sequence and often jokingly referred as "clone by phone", since the sequence can be obtained from another worker through telephone! (c) from a protein product of gene of interest – the known amino acid sequences are translated backwards to obtain the DNA sequence that encoded it.

PCR based cloning

The PCR (Polymerase Chain Reaction) based cloning is a rapid, versatile in vivo method for amplifying a specific target DNA sequence from a pool of source DNA. In order to permit the technique to function some prior information on the nucleotide sequence of target DNA is necessary. This is because the reaction has to be primed by two short stretches (15-30 base pairs) of sequences otherwise known as oligo primers. These contain the flanking sequence information of the target DNA. They are designed in such a way that in the presence of DNA precursors (dATP, dCTP, dGTP and dTTP) and heat stable DNA polymerase enzyme, new strand will be synthesized.

The output of a PCR after 30 cycles of synthesis is about 10^5 copies of target sequence. The PCR products, or amplicon can be visualized on an agarose gel as a discrete band of a particular size. The principal steps of a PCR reaction are (a) denaturation (b) annealing and (c) synthesis or extension. Technical simplicity and rapidity make PCR a very popular and central technology to genomic research.

Recommended reading

Brown, T.A., 1990. *Gene cloning - An introduction*, II Edition Chapman & Hall. 286 pp.

Griffiths, A.J., J.H. Miller, D.T. Suzuki, R.C. Lewontin and W.M. Gelbart 1996. *An Introduction to Genetic Analysis*, Sixth edition W.H. Freeman and Company. 915 pp.

Old, R.W. and S.B. Primrose 1994. *Principles of Gene Manipulation – An introduction to genetic engineering*, Blackwell Scientific Publications. 474 pp.

POSITIONAL CLONING: IDENTIFYING DISEASE GENES

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Introduction

Positional cloning refers to the cloning of a gene based on its map position. Identifying disease genes have always been a challenge in molecular genetics. This involves the identification of genetic determinants of phenotypes. In principle, the phenotype variants are the result of DNA sequence variants. Such differences in the DNA are translated to visible phenotypic changes when the 40,000 or so human genes or gene related elements are involved. A normal function of a gene can be altered either by its alterations in the levels of expression or by its abolition.

Identification of a disease gene through knowledge of the protein product

If the biochemical basis of an inherited disease is favour, it may be possible to purify and partially characterize some of the gene product. If this can be done, gene-specific oligonucleotides or specific antibodies can be generated that can be used to identify the gene.

Identification of a disease gene through knowledge of the DNA sequence

This is based on the mutations in a particular known gene as the cause of a disease. Alternatively a novel disease gene, may be identified by homology, either to a paralogous gene of the same species or to an orthologous gene in another species. An interesting application of DNA sequence knowledge is an attempt to clone genes containing expanded trinucleotide repeats.

Identification of a gene through knowledge of its normal function

Functional cloning depends on expressing random fragments of DNA in a cell or organism, and isolating any fragments that cause a desired change in function. The usual approach is a functional complementation assay, seeking fragments that correct a defect in the recipient.

If a patient has a disease gene, because of a chromosomal deletion, identifying genes present in a normal person but absent in the patient would pinpoint the disease gene. More generally, genes implicated in a disease may be expressed to a different degree in patients (this depends on the type of mutation: missense mutations alter the function but not the expression of the mRNA, but many other types of mutation result in low or absent levels of mRNA). Methods that identify the differential presence or expression of a gene therefore provide a possible route to position-independent identification of a disease gene, although more usually they are one arm of a positional candidate strategy.

Subtraction cloning can be used to select clones of the DNA that is deleted in an individual with a chromosomal deletion. Two DNA samples are compared, a normal 'test' DNA and a deleted 'driver' DNA. The test DNA is mixed with a large excession of driver DNA, denatured and reannealed by one means or other, double helixes are selected in which both strands consist of test DNA. These preferentially represent sequences in the test DNA that are absent from the driver DNA. The most celebrated application of subtraction cloning was in identifying the dystrophine gene.

Subtrative hybridisation works better with RNA than genomic DNA and subtrative hybridisation or mRNA differential display have been used to identify differentially expressed transcripts.

In positional cloning diseased genes are identified using only knowledge of their approximate chromosomal location.

Identification of a diseased gene based on no information except its approximate chromosomal location is called positional cloning. Typically these diseased genes was identified by first mapping the disease as closely as possible in affected families, and then identifying a novel candidate gene and showing that patients had mutations in that gene.

Positional cloning projects recapitulate the Human Genome Projects in miniature. In both cases the researchers first produce high resolution genetic and physical maps, then build clone contigs, before identifying and sequencing the only parts specific to positional cloning are selecting the candidate region and identifying pathogenic mutations in patients with the disease in questions. The complete sequencing of the human genome has made an enormous impact on positional cloning projects. Defining the right candidate region and testing candidate genes can still be long hard tasks but most of the intermediate stages can be achieved by intelligent use of the Genome Project data.

ENRICHMENT OF FEED INGREDIENTS THROUGH SOLID STATE FERMENTATION

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Introduction

Many process in fermentation technology have been practiced for thousands of years, like: production of cheese, fermented milk products, beer, wine and vinegar. These processes were developed from an art of 'taste' and only recently have been evolved to more technically sophisticated processes.

Solid State fermentation (SSF) refers to the controlled growth of microorganisms on moist solid substrate in the absence of free water. The free water indispensable to microbial growth is absorbed in a solid or complexed support inside a solid material. SSF can be better defined as the method of culturing microorganisms on and or within particles of a solid matrix. The liquid content, bound with them is at the level corresponding to the water activity (aw) assuring correct growth and metabolism of cells but not exceeding the maximal water holding capacity of the solid matrix. SSF, established since centuries has gained global attention, as potential biotechnological tool for the production of various substances. Some major advantages that SSF offers over submerged fermentation are in the areas of solid waste management and biomass energy conservation. Other advantages are:

- Natural habitat of the microbe can be minimized
- Lower expenditure
- Production of enzymes with higher specific activities.
- Improved enzyme stability
- Easier down stream processing and
- Generation of a protein enriched byproduct for possible use as an animal feed

The wide range of solid substrate/matrix used in SSF can be classified in three broad categories.

i) Organic materials: Organic materials are invariably polymeric molecules insoluble or sparingly soluble in water. The particles of solid substrate play a part at the same time, of support and substrates. *E.g.*: Lignocellulosic and starchy products such as sugar beet pulps, sugar cane begasses, wheat bran, wood chips, straw and so on.

ii) Mineral materials. These materials form only as support and they have to be moistened with nutrient solutions. *E.g.*: Clay granules, puzzola, perlite *etc.*

iii) Synthetic materials: Act only as support and need to be moistened. *E.g.*: Polyurethane foams, sponges *etc.*

To define the fermenter (reactor), the nature of the solid substrate used must be considered in terms of not only nutrient source for the microorganisms when it is an organic material, but also physical parameters, geometric configuration of the solid matrix, granulometry, porosity, maximum water holding capacity, resistance to compression and agitation.

The fermenter design is dependent on type of microorganism used, structure of the solid substrate, the environment conditions needed for the process and the type of use aimed at (*i.e.* research or industrial application).

Microbial growth in SSF

The solid substrate is inoculated with the desired microorganism at desired level (10^4 to 10^7 cells ml^{-1} or spores g^{-1} substrate). In case of fungi the spores germinate to form micro colonies. As it expand, the fungi quickly cover the whole substrate surface to form a mycelial layer, which increases in height and density. Fungal hyphae may penetrate into the solid particle that typically has a complex structure and complex nutritional composition. The major carbon sources are often carbohydrate, which may be soluble or macromolecular. In this case the mycelium releases enzymes, which diffuse into the substrate and hydrolyze the polysaccharide. The soluble sugars released then diffuse back to the mycelium. At the same time, oxygen is diffusing from the inter particle spaces through a stagnant gas film into the mycelial layer on the particle surface. The mycelium simultaneously consumes the soluble sugars and oxygen. As the fungal hyphae penetrate into substrate, it softens the substrate by pushing cells apart and degrading structural polymers. The degree to which this causes transformation of the substrate depends on the substrate structure and the capability of the fungi to penetrate into intracellular and intracellular spaces.

Application of SSF

1. Nutritional enrichment of agricultural byproducts/wastes

Proteins are the most important components of the food. Among the processes that can be used to supply proteins, the most important and processing are those, which are based on the microbial growth and production of microbial biomass.

The bioconversion of plant polymers (cellulose, hemicelluloses, lignin *etc.*) to protein has an increasing economic importance in many countries. Lignocelluloses can be transformed into high quality human food by feeding it to rumens. Lignification limits rumen digestibility of polysaccharides, presumably by blocking access to rumen bacteria and their enzymes to the polysaccharides. Through SSF, enrichment of protein of agricultural wastes and sub products is made possible. The SSF technology has the advantage of direct utilization of none or very few pre-treated solid substrates under aerobic conditions to produce microbial biomass products (MBP), which contain a mixture of unused substrates, cell substances of the microorganisms and external metabolites.

The nutritive value of any ingredient depends not only on the total nutrient content, but depends also on the nutrient availability and digestibility. For example, the use of bran in

human nutrition is limited by its low digestibility. The cellulose- hemicellulose matrix of the aleurone cell walls often act as a barrier to the attack on nutrients by human digestive enzymes. Moreover, anti nutritive factors originally present in the cereal grain, may limit the availability of nutrients or act as enzyme inhibitors. In particular phytic acid, the main storage form of phosphorous by interacting with cations or protonated basic residues of proteins, reduces the bioavailability of minerals and proteins. Significant reduction in phytic acid by SSF has been well documented in wheat bran by fungal phytase an enzyme that breaks down phytic acid.

The use of SSF for protein production from starchy substrates has been shown to be a feasible alternative for animal feeding. A process has been successfully developed to increase the protein content of cassava upto 20% in 30h with a suitable strain of *Aspergillus niger*. *Rhizopus olegosporus* and *Aspergillus oryzae* have also been used for the protein enrichment of cassava. *A. niger* has been reported to utilize dried citrus peel in a controlled SSF process in such a manner that the simple sugars are converted to protein fractions. Apple pomace (residue left after extraction of juice) has high acidity and though it is rich in soluble sugars, it has very small amount of proteins. It is reported that growth of yeast an apple pomace increases its protein and vitamin contents. The co-culture of *Candida utilis* and *A. niger* were found the best among several combinations which increased the protein content of dried and pectin extracted apple pomace by 20% and 17%, respectively, under SSF conditions.

The amino acid profiles of fungal protein show variations among groups of microorganisms (yeasts and filamentous fungi). On studying the application of microscopic fungi for protein biosynthesis other favourable properties of these organisms are also considered. These properties are:

i) Their ability to form an enzymic complex permitting transformation into microbial protein of various raw materials.

ii) The low content of nucleic acids in fungal biomass

Fungal culture in SSF medium affords products with protein content more than 100% higher than that in the raw material.

2. SSF of fishery wastes and agro industrial wastes

Any suitable strains of bacteria or fungi can be effectively utilized for fermentation to obtain bioenriched products. The low nutrient wastes are dried to <10% moisture content and pulverized to about 200μ size or less. The proximate composition analyses are carried out and if necessary, a combination of ingredients can be used. Moisture is adjusted to about 60-65% and pH to neutral. The aseptic substrate is then inoculated with the desired bacteria or fungi or a consortium of either (10^7 to 10^9 cell ml^{-1} for bacteria or 3-9 x 10^6 spores ml^{-1} of fungi for $10g^{-1}$ substrate) and the contents are mixed thoroughly and incubated under controlled static conditions preferably with maximum surface area for varying periods. Depending on the purpose of SSF, after desired duration, either, enzymes can be extracted from the fermented biomass or it is dried to a constant weight and used directly as a feed

ingredient. At Central Marine Fisheries Research Institute, we have undertaken fermentation of soybean flour, mixed oil cakes, soy flakes, cabbage waste and water hyacinth with both bacteria and fungi (industrial as well as local isolates). It has been observed that digestibility and feed conversion ratio has been considerably improved while using fermented products in shrimp post larval diet as fish meal substitute at 10-15% levels of incorporation. A bioenriched ornamental fish feed developed by CMFRI has been proved to be a successful alternative for imported ornamental fish feed for multispecies aquaria.

In a developing country like India, where import of costly raw materials as protein supplement for animal and aquafeed production is quite expensive, bioenrichment of cheaper ingredients through SSF would be a profitable option. Since the technology is cheaper and simple, it could be taken as a small-scale industry with a positive impact on aquafeed production.

3. Enzyme production for the food industries

A large number of microorganisms, including bacteria, yeast and fungi produce different groups of enzyme in SSF systems. It has been reported that a strain of *Aspergillus niger* produced 19 types of enzymes, -amylase was being produced by as many as 28 microbial cultures. Generally hydrolytic enzymes, *e.g.*: cellulases, xylanases, pectinases *etc.* are produced by fungal cultures, since such enzymes are used in nature by fungi for their growth. Agro industrial residues are generally considered the best substrates for the SSF processes. Some of the substrates that have been used include sugar cane bagasse, wheat bran, rice bran, maize bran, wheat straw, banana waste, apple pomace, peanut meal, coconut oil cake, wheat flour, cassava flour, *etc.* The selection of substrate depends on various factors mainly related with cost and availability of the substrate. Other factors to be considered in enzyme production are particle size and moisture level/water activity of the substrate.

The major factors that affect microbial synthesis of enzymes in SSF system include

- Selection of a suitable substrate and microorganism
- Pre-treatment of the substrate
- Particle size (inter-particle space and surface area) of the substrate
- Water content and water activity (aw) of the substrate
- Relative humidity
- Type and size of inoculum
- Control of temperature of fermenting matter/removal of metabolic heat
- Period of cultivation
- Maintenance of uniformity in the environment of SSF system and
- The gaseous atmosphere, *i.e.* Oxygen consumption rate and carbon dioxide evolution rate.

Almost all known microbial enzymes can be produced under SSF systems. Production of industrial enzymes like: proteases, cellulases, ligninases, xylanases, pectinases, amylases,

glucoamylases *etc.* and inulinases, phytases tannases *etc.* are also reported by SSF processes. The enzyme titres produced in SSF systems are many fold more than in submerged fermentations, it generates less effluents and require only simple equipments. SSF holds tremendous potential for enzyme production. The crude fermented products may be used directly as enzyme sources. In addition to the conventional applications in food and fermentation industries, microbial enzymes have significant role in biotransformation involving organic solvent media, mainly for bioactive compounds. It is hoped that enzyme production processes based on SSF systems will be the technologies of the future. Genetically improved strains would play an important role in this.

4. Production of biologically active secondary metabolites

Antibiotics: Higher yield of penicillin in a relatively shorter duration is reported. The production of penicillin ranged from 0.6 to 16.7 times relative to submerged fermentation. SSF system for production of cephalosporins with *Streptomyces clavuligerus* is well advanced. Moist barley could be effectively used for this purpose. The antibiotics, tetracycline produced by SSF was more stable than that produced by submerged fermentation and the product is able to be stored temporarily without losing activity significantly.

Other metabolites that could be produced by SSf include cyclosporin A, Iturin, ergot alkaloids, gibberellic acid (GA3) and mycotoxins.

The biosynthesis of secondary metabolites is triggered by the limitation of growth caused by the exhaustion of a key nutrient (nitrogen or phosphorous). The bosynthesis starts when the growth starts decreasing. There is no doubt that SSf offers better opportunity for the biosynthesis of low volume high cost products, *viz.* biologically active secondary metabolites. With optimization of the process and proper bioreactor, such products could be commercialized from microorganisms of different origin.

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BIOENCAPSULATION OF LIVE FEEDS

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Introduction

When aquaculture is expanding world over to meet the protein requirement of humans, the basic requirement in culture practice is the seed and major constraint noticed is in larval nutrition. The larval survival also varies with the type of organism (finfish<10%; mud crab <1%; shrimp, 20-40%; molluscs <20%). The challenge in larval nutrition lies in the fact that live feeds are not completely replaced in hatchery operations. It is believed that the optimal formulations for first feeding larvae should simulate the yolk composition and to some extent reflect the nutrient requirements and metabolic capacities of pre feeding fish or other organisms.

Bioencapsulation or bioenrichment is the process involved in improving the nutritional status of live food organisms either by feeding or incorporating within them, various kinds of nutrients. Examples of practical and experimental enrichment diets are unicellular algae, emulsion, liposomes and microencapsulated diets. Marine larvae in general require the polyunsaturated fatty acids eicosapentanoic acid (EPA: 20:5n-3) and decosahexanoic acid (DHA; 22:6n-3) for their normal development and survival. Apart from EPA and DHA, arachidonic acid (ARA; 20:4n-6) has also been recognized as essential for marine fish. ARA is the main precursor of eicasoids responsible for osmoregulation, cardiovascular functions, neural control and reproduction. The rotifer *Brachionus plicatilis* and the anostracan *Artemia* are the two organisms most extensively used as larval feed. The lipid sources in enrichment diets vary in lipid class composition, n-3 HUFA content and DHA/EPA ratio. However, EPA is present in low amounts in *Artemia* nauplii and DHA is practically absent. For this reason, the nauplii need to be enriched before they can be used for feeding marine larvae. Phospholipids from vertebrate neural tissue have high content of DHA and it has shown that deficiency during critical stages of the embryonic development may lead to neurological abnormalities. Malpigmentation observed in halibut, turbot and red sea bream, larvae have been suggested to be a consequence of sub optimal DHA content or DHA/EPA ratio in the diet. Live food enriched with essential fatty acids improved larval performance in striped bass and palmetto bass, cod, red sea bream yellow tail, milkfish *etc.* Two to four percentages are the required level of n3 (HUFA) in diet (1% EPA and 1% DHA).

The enrichment is commonly achieved by placing the nauplii in a medium, generally an emulsion, containing EPA and DHA. The nauplii are passive filter feeders and thus incorporate in their digestive tract the emulsions acting as live vehicles. This enrichment process has also been termed bioencapsulation and is successful enough to allow the use of

Artemia nauplii as larval food for marine organisms, at least during certain phases of their rearing. The success of enrichment procedure, however, is mainly due to the fact that unenriched nauplii are lethal to many larvae than to the suitability of the bioencapsulated nauplii as larval food. Although the enrichment procedure allows marine larvae to survive on a diet of *Artemia,* enriched *Artemia* nauplii are far from being an optimal diet for it as compared to marine plankton, their natural diet. The degree of success in modifying the fatty acid profile of the nauplii has shown to be influenced by the type of the enrichment diet, the enrichment conditions and the live food strain itself. Differences in enrichment conditions are related to the salinity of the culture medium, the concentration of experimental emulsion, the enrichment duration and the temperature following starvation. Also the species and geographical origin of the *Artemia* affect the success of the enrichment procedure.

Factors to be considered prior to bioencapsulation

i) Selection of the carrier or the live food, taking into account the acceptability of the organism and its size.

Commonly used carriers are:

a) Micro algae - 2-20 µ
b) Rotifers - 5-200 µ
c) *Artemia* - 200-400 µ
d) Moina - 400-1500 µ
e) Daphnia - 200-400 µ

ii) Nutritional quality, digestibility and acceptability before and after encapsulation

iii) Fixing up of the level of enrichment media to be incorporated into the carrier organism, which depends on the nutritional quality of the carrier organism before incorporation and based on feeding trials conducted in laboratory.

iv) Economic feasibility of enrichment

v) Purity of the culture of carrier organism and

vi) The easy procurement of carrier organisms, its viable culture techniques; catchability of carrier organism by target species, and its easy reproduction.

If all the above factors are satisfied, one can go for a viable and effective enrichment.

Bioencapsulation of *Artemia*

Hatching of cysts: The cysts (4 gl^{-1}) are disinfected with hypochlorite solution of 200 gl^{-1}l for 20 minutes before hatching. After washing with tap water to remove the remaining hypochlorite, the cysts (2 gl^{-1}) are incubated in filtered seawater at 28°C under continuous aeration and light. After hatching, the nauplii (<90% Instar I) are separated from the empty cyst shells and transferred to 2 L glass tubes (cylindroconical) in a water bath at 28°C with continuous aeration from the bottom of the cone using an additional air stone to keep oxygen levels above 5-6mgl^{-1}.

Enrichment with highly unsaturated fatty acids (HUFA)

Standard enrichment emulsions containing 30% and 50% of total n-3 HUFA (percentage dry matter) with DHA/EPA ratio of 0.73 and 0.84, respectively and an emulsion devoid of n-3 HUFA. The emulsion contains (percentage wet weight) lipids (62%), water (30%), emulsifiers, antioxidants and liposoluble vitamins (A, D, E And K). The enrichment conditions are standardized at 28°C and salinity at 34 parts per thousand, for a period of 24h. The larvae are then cleaned in water and 28.9mg g^{-1} enrichment (DHA) could be obtained with 50% n-3 HUFA emulsions at 0.39 l^{-1}.

Enrichment of *Artemia* nauplii with free amino acids (FAA)

Amino acids are the major substrates of aerobic metabolism during the development of embryo and yolk sac larvae of marine species, which have pelagic eggs. Due to rapid larval growth and development, there is large amino acid requirement both to maintain the appropriate concentration in the tissues necessary to obtain an optimal growth rate and amino acid utilization, and to supply the fuel for energy metabolism. The need for dietary free amino acids for marine fish larvae is also suggested by the large pool of free amino acids found in marine invertebrates such as copepods, which are natural food for the larvae in the sea. *Artemia* contain lower level of free amino acids, especially methionine. This suggests that methionine is a limiting amino acid for fish larvae when fed *Artemia* nauplii.

One approach to enrich *Artemia* with water-soluble substances, such as FAA is to use liposomes. Liposomes are spherical vesicles (10nm-20μm) in which an aqueous volume is entrapped by a membrane composed of lipid molecules, usually in the form of phospholipids. These are potent delivery vectors for hydrophilic as well as hydrophobic nutrients and are potential carriers for FAA. Phospholipid form concentric bilayers when dispersed in an aqueous medium enclosing the aqueous material in the core, as well as within the bilayered lamellae. The compatible size range and complete digestibility of liposome make them a good vehicle for the study of nutritional requirements of aquatic filter feeders.

Liposome preparation

Egg yolk phospatidyl choline (PC): cholesterol (10:4 w/w): 100 mg egg yolk and 40mg cholesterol are dissolved in 5 ml chloroform: methanol (2:1v/v) in a round bottom flask. The solvents are evaporated at 30°C under nitrogen in a rotary evaporator and the resulting lipid film is further dried for 30 min in vacuum desiccators. 5 ml of 530mv NaCl solution is introduced into the flask along with 5 ml of 300mM methionine and rotated slowly for 30 min at 35°C. The resulting liposome suspension is extruded through a polycarbonate filter (0.6μm). Liposomes can provide protection against degradation, as well as easier incorporation of the lipids contained in them.

Encapsulation protocol

Artemia cysts are hydrated, decapsulated and incubated at 28°C under continuous aeration and illumination. After 24 h, larvae are washed and stored at 20°C for 5h before transferred to 50ml plastic centrifuge tubes for enrichment. Add 0.625ml of 300 mM methionine and equal amount of methionine in their respective liposome suspensions also. After 16 h, the nauplii are washed with tap water and transferred to seawater.
Direct enrichment: In this procedure, nauplii are enriched with methionine directly dissolved in the culture water and the nauplii are analyzed for FAA after 16h.

Nutritional improvement of rotifers

Improvement of rotifer *Brachionus plicatilis* as food for marine fish larvae has been achieved through enrichment with various diets containing different levels of (n-3) highly unsaturated fatty acids (HUFA). These diets include microalgae, lipid emulsions, fish oils, microparticles and microcapsules containing lipids. HUFA content of the enriched rotifers is to a great extent a reflection of the content of these fatty acids in the diets. Enriched rotifers have been shown to be able to maintain their improved nutritional value for several hours at 10°C during their application as live food for marine fish larvae.

The concept of long-term (LT) and short-term (ST) enrichment is also done for live feed enrichment. For LT, it combines growth and n-3 HUFA enrichment during the production phase. The ST enrichment involves short (<24h) exposure to high concentration of specific HUFA rich feed following culture. The latter is important for cold-water fishes.

Short-term enrichment of rotifers with microalgae

Microalgae are used for short-term enrichment of rotifers before they are given to the fish larvae to improve the content of n-3 fatty acids in the rotifers. Short-term feeding with algae affects the protein content and dry weight of the rotifer.

Preparation of enrichment

For emulsified lipids, the (n-3) HUFA concentration in the lipid source should be very high.

- 5g fish oil is homogenized for 2-3 min in a homogenizer or mixer or by vigorous shaking.
- Observing under microscope ensures proper emulsion
- Stored under refrigeration till use.
- Emulsifiers may be added to maintain the emulsion or a strong shaking prior to use reforms the emulsion.
- The enrichment media may be supplemental with water and fat-soluble vitamins like vitamin A, D, E and K to homogenization.

One potential risk in using fish oil for HUFA enrichment, the n-3 HUFA particularly DHA in the very small triacyl glycerol (TAG) micelles generated in enrichment procedures are prone to auto oxidation, especially under continuous aeration employed, for prolonged periods. The addition of natural antioxidants vitamin E and C (generally added as oil soluble α tocophenyl acetate and ascorbyl palmitate) which are not effective until hydrolyzed in the intestinal tract and absorbed and or synthetic antioxidants like ethoxyquin or butylated hydroxyl anisole (BHA), minimizes peroxidation.

Lecithin can be used to considerable advantage in enriching the nauplii with 22:6n3 HUFA rich fish oils (90:10; Fish oil: lecithin), lecithin acting both as natural emulsifying agent and as a natural protectant against peroxidation. Oils from marine organisms like heterotrophic dinoflagellate *Crypthecodinium cohnii*, which is mass cultured for a triacyl glycerol rich in 22:6n-3. This oil is used to supplement infant formulae. Frozen-thawed cells of *C.cohnii* have recently been used very successfully to supplement *Artemia* nauplii directly with 22:6n-3. Spray dried *Schizochytrium* sp. a single celled heterotrophic marine proteist similar to water molds of group *Labyrinthulomycota* is also used.

Bioencapsulation with vitamin C

Bioencapsulation is done using the lipophilic ascorbyl palmitate (AP) as a stable and bioavailable source of Ascorbic acid (AA) in emulsions and particulate boosters for the live food prior to their feeding to fish larvae. Applying experimental self-emulsifying concentrates supplemented with 10 and 20% AP, high levels of AA could be obtained in 24 h enriched *Artemia* nauplii. Four fold increase in AA is obtained with 20% AP.

Bipencapsulation with drugs

In addition to the use of enrichment in nutrients, live food mediated delivery of therapeutic drugs has emerged as a new tool for disease treatment in larval culture. This is normally done through liposomes.

The overall nutritional quality of the live food organisms depends on the content and nutritional balance of carbohydrate, protein and lipids. The content of essential amino acids and protein in live food must meet the requirement for growth and cell maintenance of fish larvae. The enriched live food might be starved in the culture tank before they are consumed by the larvae and in this period, the protein, lipid and fatty acids decreases. The loss of all these components increase with increasing water temperature. The total lipid content loss is at higher rate than the protein content. Highest loss rate is for DHA and considerably lower for EPA.

It is concluded that survivability of marine larvae in hatcheries depends on the larval nutrition, which in turn is due to the quality loss (potency loss of the stock on continuous use, mutation *etc.*) of live food organisms. Bioencapsulation can be effectively and successfully employed to cut down the larval mortalities in hatcheries.

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AQUAFEED BIOTECHNOLOGY-AN OVERVIEW

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Introduction

Aquafarming is emerging as a successful small-scale bio-industry in India. In order to sustain this industry an organized feed industry is essential to manufacture and supply quality controlled compounded feeds for the different stages and production systems The exogenous feeds used in aquaculture can be broadly grouped into (i) supplementary feeds and (ii) complete feeds. Supplementary feeds, by and large are empirical formulations, which do not provide the essential nutrients in adequate levels and balanced proportions as in the case of complete feeds. Supplementary feeds can be either made of a single feed ingredient or a mixture of ingredients; but usually low-cost agricultural and animal wastes, and by-products are used. Thus, the production achievable through supplementary feeding depends on the quality and quantity of the supplementary feed and on the natural food produced in the ponds.

In the semi-intensive and intensive systems of fish and crustacean culture provision of nutritionally balanced complete feeds is essential to achieve maximum production. In these systems feed forms the major operational input and often accounts for 40 to 60 per cent expenditure. Therefore, it is of foremost importance that the feeds are scientifically formulated to provide all the essential nutrients and energy in adequate levels and balanced proportions, optimally processed and judiciously supplied to achieve maximum efficiency at optimal cost.

Need for ecofriendly feeds

Feed holds the key not only for successful aquaculture but also for its sustainability. For instance, until the late 1980s choice of shrimp feed was primarily based on its cost, potential FCR, growth and production potential. Concerns of environmental degradation, disease outbreaks, mortalities, and poor growth and production in the 1990s have posed new challenges to aquafeed industry and aquaculture. Wastes derived from unconsumed food, faeces and metabolism of nutrients are the major contributors to pollution in poorly managed farms. These wastes are excellent nutrient media for the propagation of microbes, some of which are pathogenic while others like luminescent bacteria produce toxins which induce stress, and predispose the shrimp to diseases, cause reduced growth and production from farms. Excessive waste nutrients in turn cause plankton blooms, drastically alter the dissolved oxygen profile in shrimp farms due to high BOD and COD, and produce toxic gases like H_2S.Waste production is related to the performance of a feed in culture and feed conversion ratio (FCR) could be used as an index of the feed based wastes (Table 1). A rough estimate of the waste production from shrimp culture is given in Table 2. In a

four-month crop about 47 % of the organic matter, nitrogen and phosphorus wastes are generated during the 4th month of feeding. Thus it is imperative to produce less pollutive, quality assured feeds and evolve eco-friendly feed management strategies for culture.

Characteristics of eco-friendly feeds

Feed quality is defined by the physico-chemical characteristics matching the specific requirements of the grow-out stages at optimum intake, with minimum output of feed, faecal and metabolic wastes. The factors, which determine the quality of a feed are its nutrients profile, anti-nutrient status, particle size, texture, stability of nutrients, attr[illegible] digestibility, [illegible] and shelf-[illegible] on [illegible] nutritionally adequate feeds requ[illegible] ation, quality control and biological evaluation. A well balanced diet not only results in higher production but also provides the nutrients necessary to hasten recovery from diseases or aid the animal in overcoming the effects of stress. Design and fabrication of complete artificial diets need information on a variety of biological, physiological, biochemical and nutritional aspects, besides the intensity of culture operations. Information on the dietary levels of essential nutrients that promote maximum growth at optimum intake, the preferred form of diet, feeding behaviour, role of attractants, feeding stimulants and deterrents, digestive capabilities of the species and also about the feed ingredients and additives that should go onto the feed for proper ingestion and utilization by the animals is required.

Balance of nutrients

A balanced feed formula should include an energy source plus sufficient indispensable amino acids, essential fatty acids, phospholipids, specific vitamins and minerals to sustain life and promote growth. In the case of crustaceans, a source of sterol is also necessary. One of the major constraints in developing high performance eco-friendly feeds is the non-availability of complete data on quantitative nutrient requirements for the different stages.

The nutritive value of a dietary protein is dependent on its amino acid profile and their bioavailability. All the essential amino acids, viz., arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine should be found in adequate levels and balanced proportions. Amino acid imbalance besides affecting growth and feed efficiency, leads to increased production of nitrogenous wastes mainly in the form of ammonia. Similarly excess of protein in the diet, more than necessary to sustain protein synthesis, also results in nitrogenous waste production.

Nitrogen is the major source of waste derived from protein in the food. Reduction in nitrogen output could be achieved by optimum use of amino acid-balanced protein, by maintaining optimum protein-energy ratio and by ensuring balance of non-protein nutrients. Amino acid imbalance in the diets can also occur during processing of feed proteins and manufacture of feeds. Excessive heat treatment markedly reduces protein digestibility and biological value due to the destruction of amino acids by oxidation through the formation of linkages between individual amino acids, which are more

resistant to digestion. Bioavailability of lysine and methionine are markedly affected by improper processing. Heat treated fish meals containing free histidine and histamine have decreased EAA availability. There are also reports of antagonism between amino acids when they are found in disproportionate levels. Leucine/isoleucine antagonism as well as arginine/lysine antagonism are well established. Even when a protein has well balanced amino acid profile, its utilization can be affected if protease inhibitors are present in the ingredients used.

It is therefore necessary to prepare a protein-mix, which satisfies the aminoacid needs of the species. The levels of digestible energy, essential fatty acids, vitamins, and anti-metabolites also significantly influence the protein utilisation and nitrogen waste output.

Crude fibre levels should be carefully monitored and should be maintained within optimal levels. High fibre levels offer low food retention time in the digestive tract for the enzymes to effectively digest the ingested food. Thus a good proportion of the food is excreted as faeces, in high fibre diets.

Quantitative requirements of most of the vitamins have not been clearly defined for shrimps. Vitamin premixes currently in use are mostly empirical formulations. Water soluble vitamins as a group need monitoring as some of the vitamins leached from the diet, and unconsumed feed promote the growth of phytoplankton and microbes, occasionally causing blooms. Thiamine, biotin, and cyanocobalamin have been known to trigger blooms.

Mineral and trace element requirement of shrimps are also not well established. Phosphorus is considered to be the dominant element in the feed derived wastes being about 86 % in shrimp culture. Dietary source and level of phosphorus affect its utilisation by shrimps. Phosphorus from plant ingredients and tricalcium phosphate are poorly utilised. Eco-friendly feeds should contain optimum level (0.9%) of bioavailable phosphorus. Excess of trace elements such as Zn, Co, Fe, Mn in the diets, when excreted also trigger phytoplankton blooms as they are essential for growth and multiplication of phytoplankton.

Anti-Nutrients

Several anti-nutrients (toxic principles) occur in some of the raw materials used in feed formulation (Table 4). Gossypol, Cyclopropenoic acids, thioglutcosides, cynogens, hemaglutinin, phytic acid, mycotoxins, antivitamin, antimineral and antienzyme inhibitors fall under this category. Inclusion of ingredients containing any of the toxic principles could affect the food intake, bioavailability of nutrients and also nutrient metabolism and cause physiological and metabolic disorders. Such ingredients should either be excluded from the diet or added after adequate preprocessing to remove the toxic principles, thereby minimizing wastage of feed.

Additives

Binders, antioxidants, antimicrobial agents, attractants, feeding stimulants, growth promoters, pigments and therapeutants are essential in feed to meet specific needs. Judicious use of these additives either directly or indirectly helps to reduce feed derived wastes in shrimp culture. However, therapeutants, especially antibiotics should be used with utmost care adhering to prescribed dosage and schedules. Immuno-modulators such as β-glucans, lecithins, lipopolysachrides and other bioadditives, which have been shown to enhance disease resistance should be used in disease management programmes.

Quality of Raw Materials

The quality of a feed is greatly determined by ingredient. Ingredients with high levels of non-protein nitrogen, crude fibre, ash, salt content, oxidized lipids and anti-nutritional factors should be excluded from shrimp feeds.

Processing

Microgrinding and homogenous mixing of ingredients, premixes and additives, optimum conditions of pelleting, cooling and drying are very important for proper utilisation of the feed by shrimps and to minimize feed waste. Microgrinding makes available more surface area for enzymatic action and hence improves digestibility of the feed. Fine grinding also helps in better gelatinization of starch and binding. Mixing processes should ensure uniform dispersion of the macronutrients and additives so that each unit weight of the finished feed has a balanced nutrient profile.

Fish feeds may be pelleted by what is sometimes called the "extrusion" process, thereby expanding rather than compressing the various ingredients. Variations in formulation and processing allow a wide range in bulk density; 0.25. - 0.3 g/cc being common for fish feeding. This feature makes them attractive for certain types of fish culture. Fish may be observed while eating and the amount of feed limited to that which is accepted. The number of fish in a water impoundment and their health can be observed without sampling. In general, the processing of expansion of extrusion consists of: (a) conditioning soft feed which is in meal form to contain 25-30 percent water, (b) conveying this conditioned feed by auger into a pressure-sealed cylinder, (c) injecting steam, thus decreasing friction of material within the cylinder and increasing gelatinization of raw starch, (d) extruding to atmospheric pressure, almost exploding the material through holes in the die plate at the end of the cylinder, (e) cutting off the extruding ribbon at the outside of the die plate by a rotating knife, and (f) drying the pellets in a high-temperature oven at about 120 C to a moisture content suitable for storage.

Several details should be added to this general process description for a better understanding of floating pellets. The feed formula is important in obtaining a desired

expanding texture. Cereal grains can be expanded to a very low bulk density, whereas protein concentrates low in starch may remain unaltered in bulk density. Raw starch is a requirement of a good floating pellet; 90 percent being gelatinized during the 30 to 60 seconds the feed is in the expander cylinder. Pressure builds up to several atmospheres due to forward passage of the material into a smaller volume. Superimposed steam causes a high product temperature, changing the consistency of the material from a free-flowing meal to a dough. By the sudden release of pressure at the discharge end, the feed assumes a "puffed rice" texture like some breakfast foods and snacks.

Following oven drying, a standard pellet cooler is often used to lower product temperature after internal moisture is less than 13 percent. Even with this treatment, previous high temperatures partially destroy heat-labile vitamins and decrease the availability of some amino acids. Rather than over-fortifying the formula before pelleting, as is done for preparation of hard pellets, heat-sensitive additives may be sprayed onto expanded pellets after extrusion.

Biological tests under pond conditions with natural food available have shown no difference in growth of catfish and goldfish using a formula feed processed by hard pelleting and by the extrusion process. Also no significant difference was found chemically in total crude protein or in individual amino acids for several formula feeds each prepared as hard pellets and as extruded pellets. However, testing fish growth in tanks and cages has revealed a partial destruction of some nutrients in extruded feeds.

There is evidence that fish fed with floating pellets contain more liver and body fat than those fed the same formula feed processed as a hard pellet. This may be due to the increased digestibility of the carbohydrate part of the ration. The expansion process is expensive compared with other methods of feed manufacture in terms of equipment cost, heat used in generating steam and in oven-drying, and in loss of nutrients. It may be concluded that the value of extruded pellets-is best measured by practical feeding tests using data on feed conversion, cost of management, and acceptability by fish as deciding factors.

Feed Particle Size

Feed particle size is very important for proper utilisation. Particles should be adequately sized to suit the mouth parts and digestive capabilities of the animals. Under-sized and [illegible] particles should be screened before feeding to prevent feed wastage, degradation of the culture environment, and propagation of pathogenic organisms.

Digestibility

Shrimps have a very small digestive tract, which is capable of utilizing soft food material, similar to that of live food, which they consume in nature. Artificial dry diets should have rehydration characteristics and render flexibility for feeding by the shrimps. Addition of exogenous enzyme or inclusion of enzyme or bio-preprocessed ingredients would further improve the digestibility of nutrients.

Shelf-life

Prolonged storage lowers the biological value of feeds. Shelf-life is also dependent on the shelf-life of ingredients used in the feed. Ingredients and finished feeds should be used as fresh as possible. Prolonged storage alters the quality due to enzymatic action and chemical changes. Chemical changes included breakdown of lipids, formation of free fatty acids and rancidity. Rancidity reduces the palatability of the feed and the availability of amino acids, and certain vitamins. Raw material and compounded feeds being highly nutritious attract insects such as beetles, weevils and moths, which expose the feed to fungi and introduce contaminating bacteria. Fungi produce highly potent mycotoxins like aflatoxins, which are toxic to shrimps. Vitamin potency decreases during storage. Feed stored for periods exceeding six months need thorough chemical and biological evaluation for acceptability and digestibility.

Eco-Friendly Feed Management

Judicious feed management is also an important factor in achieving good feed efficiency and reducing feed wastage. Waste production could be reduced by selecting feeds which are freshly made, quality assured, proven with best potential FCR, rejecting feeds which have lost their nutritional potency and have poor acceptability to shrimp and poor stability when introduced into water, by using feed of appropriate particle size designed for a particular stage, and by regulating the ration and feeding schedules with reference to feeding guides, response of the animals to the feed and environmental conditions.

Table 1: Relationship of FCR to waste production per tonne of shrimp

FCR	Organic Matter (Kg)	Nitrogen (Kg)	Phosphorus (Kg)
1.0	500	26	13
1.5	875	56	21
2.0	1250	87	28
2.5	1625	117	38

Table 2: Rough estimate of the feed derived waste production/crop/ha
(Production 2 tonnes/ha: FCR 1.5)

	1st M Kg.	2nd M Kg.	3rd M Kg.	4th M Kg.
Feed offered	120	510	960	1410
Waste production				
Organic matter	70	298	560	822
Nitrogen	5	20	36	52
Phosphorus	2	7	14	20

Biotechnological interventions:

Biotechnological interventions in aquafeed development include nutrient enrichment through solid state fermentation; production of B group vitamins, essential amino acids like lysine, novel methods of production of essential fatty acids like docosahexaenoic, eicosapentaenoic and arachidonic acids, and carotenoids. Microbial and enzyme mediated bioconversion of raw materials have been shown to improve digestibility and nutrient bioavailability and reduction in waste output. The most significant impact is in breakdown of antinutrients and fibre levels in plant products.

Conclusion

There is increasing concern worldwide about the decline in world fishmeal production and the availability of adequate quantities of animal-based raw materials and key micro ingredients for meeting the demands of the growing aquafeed industry. In future biotechnological interventions will have greater role in aquafeed formulation and production for sustainable fish and crustacean aquaculture.

PROBIOTICS AND ITS APPLICATION IN MARICULTURE

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Introduction

Aquaculture is a multidisciplinary activity, more complex than agriculture due to the multidimensional aquatic medium. The aquaculture boom and increased socio-economic benefits together with increase in extent and intensity of aquaculture are alleged to have created several problems, particularly those of deteriorating water and soil quality and outbreak of diseases (Kutty, 1999). For example, the global production of farmed shrimp has doubled in the past 15 years. India has also witnessed such spectacular growth in shrimp farming until the mid nineties, after which it became beset with disease and environmental problems. Overstocking, overfeeding and excessive use of antibiotics during farming are some of the reasons attributed to the outbreak of shrimp diseases. Even by the year 2001, a total solution to the problem has evaded the scientists and policy makers. But what has been recognised now by scientists and policy makers is that aquaculture must be environmentally friendly so that it can sustain itself without seriously affecting the coastal ecosystem. In other words, the ecological footprint of aquaculture must be sufficiently small so as to ensure sustainability.

In the recent past, the farm animal production industry in the west was also plagued with disease outbreaks resulting in excessive use of antibiotics. The resulting residual antibiotics in meat products lead to curbing of its usage in animal rearing and production. The farmers then turned to an age-old practice of using beneficial bacteria to quell infectious diseases. Thus, Parker (1974) introduced the modern concept of probiotics more than 25 years ago. Aquatic animals are quite different from land animals for which the probiotic concept was developed, and therefore, the probiotic usage in aquaculture, especially in shrimp culture has taken a different meaning. The high risk of losing their crop to disease attack has prompted many shrimp farmers all over the world, and especially in India, to use probiotics during their culture operations.

In this paper, an attempt is made to review the state of probiotic usage in aquaculture, particularly shrimp farming. The concept of probiotics as used in animals and humans and the manner in which it is differently employed in the aquatic environment will be examined in detail. A pointer to the future research needs in this direction will also be attempted.

What are probiotics?

The origin of the term probiotic is attributed to Parker (1974) who defined them as organisms and substances, which contribute to intestinal microbial balance. However, the concept of microbial manipulation was first appreciated by Metchnikoff during the early 1900s when he viewed the consumption of yoghurt by Bulgarian peasants as conferring a long span of life. Although evidence for a link between longevity and ingestion of fermented milk products has not been proven yet, some workers have claimed that its therapeutic value is related to viable bacteria, in particular *Lactobacillus* sp. Although a strict definition of probiotics is difficult to come by, Tannock (1997) proposed it as "*living microbial cells administered as dietary supplements with the aim of improving health*". Gatesoupe (1999) reviewed the state of probiotic usage in aquaculture and stated that the first application of probiotics in aquaculture is relatively recent, but the interest in such environmentally friendly treatments is increasing rapidly.

There now exists a growing number of scientific papers, which deal specifically with use of probiotics in aquatic animals. Yet, more questions have been raised as to whether probiotics have any relevance in the aquatic environment (Gatesoupe, 1999). Aquatic animals are quite different from land animals for which the probiotic concept was developed. Live-bearing endotherms undergo embryonic development within an amnion, whereas the larval forms of most fish and shellfish are released into the external medium at an early ontogenetic stage. Thus the latter are exposed to all types of microflora available in the medium, while the former develop a particular type (obligate or facultative anaerobes) of gastrointestinal microbiota. Most identified probiotics belong to the dominant or sub-dominant genera of *Bifidobacterium*, *Lactobacillus* and *Streptococcus*. On the other hand environmental microbes like *Vibrio* and *Pseudomonas* are the most common genera in crustaceans (Moriarty, 1990), marine fish (Sakata, 1990) and bivalves (Prieur et al., 1990).

As compared to terrestrial livestock where resident microbes benefit from a fairly constant gastrointestinal habitat, the intestinal tracts of aquatic animals have microbes that are transient (Moriarty, 1990). Aquatic animals being poikilotherms, their gut-associated microbiota varies with temperature (Liesel, 1990) and salinity (Ringo and Strom, 1994). The continuous water flow increases the influence of the surrounding medium, in much the same way as the effect of water flow observed in filter feeders like bivalves, shrimp larvae and live food organisms (Gatesoupe, 1999). The environment and the food eaten play a key role and thus, in bivalves (Sugita et al., 1981; Prieur et al., 1990) and in penaeids (Moriarty, 1990) the associated microbiota is very similar to those found in seawater and sediment. In larval and juvenile fish, the influence of food on gut microflora has been clearly demonstrated (Ringo et al., 1995; Gatesoupe, 1999). Similar conclusions on crustaceans, especially penaeids are yet to be made although the influence of bacteria brought through live food organisms is well known.

Types of Aquatic Probiotics

Recognising the conceptual difference of terrestrial and aquatic probiotics, Gatesoupe (1999) suggested a modification in the definition of probiotics as used in aquaculture. He defined probiotics as - *microbial cells that are administered in such a way as to enter the gastrointestinal tract and to be kept alive, with the aim of improving health.* He further classified (Fig.1) the microbial preparations used in aquaculture into 3 types – biocontrol agents, probiotics and bioremediation agents. Biocontrol agents are those methods of treatment using the antagonism among microbes to kill or reduce the number of pathogens in the aquaculture environment (Maeda et al., 1997). Those bacterial treatments which improve the water quality and thus indirectly the production were termed as bioremediation agents.

The bioremediation agents have also been termed as bioaugmentation agents or water additives (Moriarty, 1998) and probiotics. Porubcan (1991a) reported the increase in yield and survival of *Penaeus monodon* by using floating biofilters pre-inoculated with nitrifying bacteria. These helped to decrease the amount of ammonia and nitrite in the rearing water. He (Porubcan, 1991b) further reported that the introduction of *Bacillus* spp. in proximity to pond aerators reduced the chemical oxygen demand and increased the yields. Recently, several commercial products have sought to exploit the idea that bacteria, which improve water quality, may be beneficial to animal health. Among shrimp farmers in India, these products are known as *water-probiotics* and most of them contain nitrifying bacteria and/or *Bacillus* spp. The nitrifying bacteria have strict ecological niches, and they have not been detected in the gastrointestinal tract of animals (Gatesoupe, 1999). *Bacillus* spp. are not autochthonous in the gastrointestinal tract, but they have been isolated from fish (Kennedy et al., 1998; Sugita et al., 1998), crustaceans (Austin and Allen, 1982; Sharmila et al., 1996), bivalves (Sugita et al., 1981) and shrimp larval rearing medium (Mohamed, 1996; Rengpipat et al., 1998). Many of these *Bacillus* spp. strains have antibiotic properties and may be active during intestinal transit.

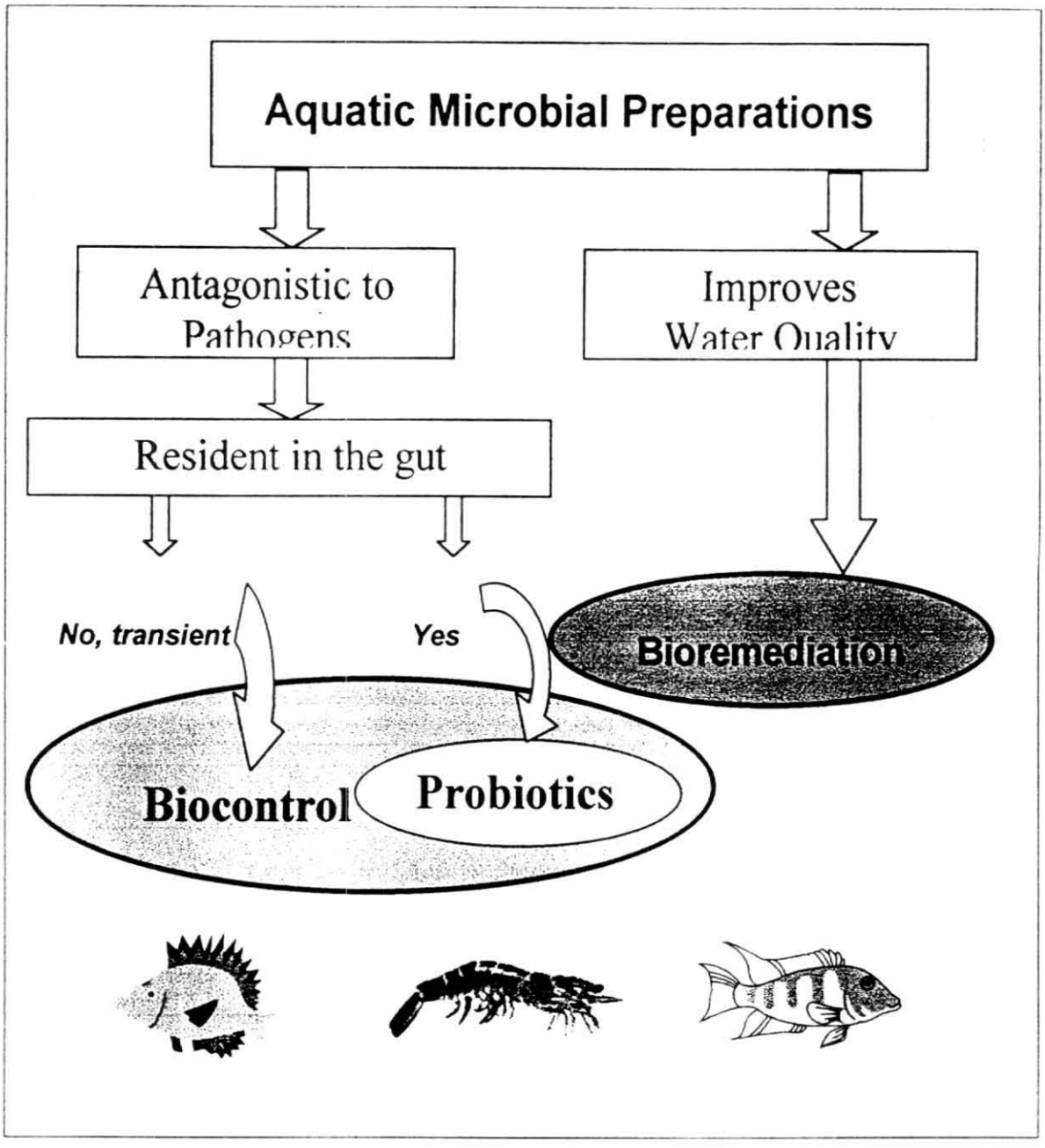


Fig. 1. Classification of microbial treatments used in aquaculture according to current terminology modified from Gatesoupe (1999).

It is important to clear the concepts and definitions with regard to the term probiotics. At present the definitions and classifications brought forth by Gatesoupe (1999) serve the purpose and can be applied without confusion in shrimp culture. The commercial availability of probiotics and bioremediation agents in shrimp culture and its widespread usage in India has spawned separate terminologies among shrimp farmers. The strict probiotic agents are known as gut-probiotics and the bioremediation agents are known as water-probiotics.

Modes of Action

Probiotics

Several mechanisms have been investigated whereby bacteria could function as a probiotic. These include adhesion to digestive tract wall to prevent colonisation of pathogens (competitive attachment), neutralisation of toxins, bacteriocidal activity and increased immune competence. The experimental introduction of lactic acid bacteria (LAB) into the intestine of fish has already been reviewed (Ringo and Gatesoupe, 1998). Several studies have shown that it is possible to maintain artificially the LAB population at high levels by regular intake through feed especially in cod, salmon and turbot. Such studies on tropical fish species and crustaceans are lacking. Adhesion is acknowledged as the first step of a microorganism in the process of colonisation and the intestinal mucous plays a vital role in this process. There are reports in endothermic animals that there is a certain degree of host-specificity in the adhesion process. Adhesion to intestinal mucous has also been assayed in vitro in fish (Jobom et al., 1997). Intestinal bacteria of turbot adhere specifically to intestinal mucous than to any control surface. Specific adhesins have been demonstrated in the adhesion of yeasts to intestinal cell walls of rainbow trout (Vazquez-Juarez et al., 1997). Similar studies on crustaceans in general and shrimps in particular are wanting.

Production of lactic acid by lactic acid bacteria reduces the pH of the stomach contents in endothermic animals. Studies in vitro have shown that an acid condition of less than pH 4.5 prevents the growth of many bacteria including coliforms, but still allows the growth of some strains of lactobacilli (Sissons, 1989). LAB are also known to produce hydrogen peroxide which has bacteriocidal actions in vitro and produces a metabolite thought to neutralise the effect of enterotoxin released from coliforms. Clearly LAB is the best studied among all probionts, even so, the effect of its use in crustaceans has not been studied. Although LAB is not the dominant gut microflora in marine fish as compared to endothermic animals, researchers have been able to introduce LAB into larval and juvenile fish with pronounced protection against *Vibrio* infections. Uma et al. (1999) reported that the growth and survival of *P. indicus* juveniles were significantly improved by the addition of Lacto-sacc™ (a commercial livestock probiotic feed supplement composed of *Saccharomyces* sp., *Lactobacillus acidophilus* and *Streptococcus faecium*) at levels ranging from 2.5 to 7.5 g/kg basal feed. A challenge with *Vibrio alginolyticus* resulted in low mortality rate in Lacto-sacc™ fed animals than in control group. However the presence of these organisms in the gut of the shrimp after feeding was not ascertained. In a more recent study, Sridhar and Raj (2001) introduced strains of *Bacillus* and *Micrococcus* isolated from shrimp guts by coating them on compounded diets into *P. indicus* post-larvae. They observed significantly higher specific growth rates and survival in treatment groups than control. Upto 10^6 cfu/shrimp of probiotic organisms was detected in the gut of the post-larvae. Similar introductions are yet to be made with other marine shrimps, yet, it must be borne in mind that Lactobacilli have never been isolated from shrimp guts.

Enhanced immunity by probiotic treatment has been well demonstrated in endothermic animals. For example, raised activities of macrophages and lymphocytes in mice following oral inoculation with LAB was observed implying an immunopotentiating role for LAB in the gut. Itami et al. (1998) reported the enhancement of disease resistance of *Penaeus japonicus* after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. They reported that that the phagocytic index and survival after challenge with *Vibrio penaeicida* and white spot syndrome baculovirus of shrimps fed with peptidoglycan was significantly higher than that of control. The immune effects of probionts in crustaceans is an area in which there is little work and needs immediate attention.

Biocontrol Agents

Antagonism to pathogens is a characteristic of a good aquatic biocontrol agent. Antagonism may be mediated not only by antibiotics, but also by many other inhibitory substances like organic acids, hydrogen peroxide and siderophores (Gatesoupe, 1999). These compounds produced by the biocontrol agents are highly dependent on experimental conditions that are different in vitro and in vivo conditions. It was Maeda and Liao (1992) who first isolated a strain "PM-4" (subsequently identified as *Thalassobacter utilis*) from the rearing water of larval *Penaeus monodon* for use as a biocontrol agent. This strain increased the survival rate of the larvae of *P. monodon* and the swimming crab *Portunus trituberculatus* and repressed the growth of *Vibrio anguillarum* (Maeda et al., 1997; Nogami et al., 1997). Mohamed (1996) used several strains of heterotrophic bacteria as feed for *P. monodon* larvae and found that a strain of *Pseudomonas* increased the percentage survival and a strain of *Micrococcus* increased the metamorphic rate to PL-1 stage. Haryanti et al. (1998) reported the increased survival of *P. monodon* larvae on rearing with a strain "BY-9" which also inhibited the growth of *V. harveyi*.

In Ecuadorian shrimp hatcheries Griffith (1995) reported the control of *Vibrio parahaemolyticus* associated outbreak of vibriosis through the artificially increasing the proportion of *V. alginolyticus* in the rearing medium. Hatchery down time was reduced from approximately 7 days per month to less than 21 days annually, while production volumes increased by 35% and overall antibiotic use was decreased by 94% between 1991 and 1994. Further, Griffith (1995) found that the survival, production, feed conversion and growth rates in the farm were not negatively affected by the use probiotic fed larvae, on the contrary, they were even improved by their application. Table 3 lists the various antagonist agents tried in crustaceans, its source and effect. It can be seen that some bacteria are even antagonistic to viruses and they may even work as a biocontrol agent for viral diseases.

Table 3. Antagonism of aquatic microbes isolated from crustaceans and its effect (modified from Gatesoupe, 1999)

Biocontrol Agent	Source	Tested Against	Effect	Reference
Alteromonas sp	*Palaemon macrodactylus*	*Lagenidium* (fungus)	Protection of crustacean embryos from fungal infection	Gil-Tunes et al., 1989
Alteromonas-like	Shrimp hatchery	*Vibrio* sp.	Protection from vibriosis	Tanasomwang et al., 1998
Pseudo-alteromonas undina	Seawater	*V. anguillarum* IHNV	Increase in growth and survival of larvae	Maeda et al., 1997
Thalassobacter utilis	*P. monodon*	*Haliphthoros* sp. (fungus)	Increase in survival of larvae	Nogami et al., 1997
T. utilis	*P. monodon*	*V. anguillarum*	Increase in survival of crab larvae	Nogami and Maeda, 1992
V. alginolyticus	*P. monodon*	*V. harveyi*	Inhibitory effect	Ruangpan et al., 1998
V. alginolyticus	Shrimp hatchery	*Aeromonas salmonicida*, *V.anguillarum*, *V. ordalli*, *Yersina ruckeri*	Increased resistance of salmon against experimental infections	Austin et al., 1995
Vibrio sp.	Shrimp hatchery	IHNV, OMV	Effect against fish viruses	Direkbusarakom et al., 1988
V. alginolyticus	Shrimp hatchery	*V. para-haemolyticus*	Increase in shrimp larval survival	Griffith (1995)
Pseudomonas sp., *Micrococcus* sp.	Shrimp hatchery	Not determined	Increase in shrimp larval survival and metamorphic rate	Mohamed (1996)

In another recent study by Rengpipat et al.(1998) *Bacillus* S11 bacterium isolated from tiger shrimp habitats in Thailand was added to shrimp feed in three forms: fresh cells, fresh cells in normal saline and a lyophilized form. After a 100-day feeding trial with probiotic supplemented and non-supplemented (control) feeds, *P. monodon* (from PL 30) exhibited significant differences ($p<0.05$) in growth, survival and external appearance

between probiotic and control groups. There were no significant differences among the three treatment forms. After challenging the shrimps with a shrimp pathogen, *Vibrio harveyi*, by immersion for 10 days, all probiotic treatment groups had 100% survival, whereas the control group had only 26% survival. The main bacterial flora in control group shrimp guts was *Vibrio* spp., while those in all treatment groups were mostly *Bacillus* S11. This kind of bacterial species replacement was also observed in the rearing medium and faeces. However, whether the *Bacillus* S11 was able to colonise the gut even after stopping the probiotic feeding was not investigated.

Bioremediation Agents

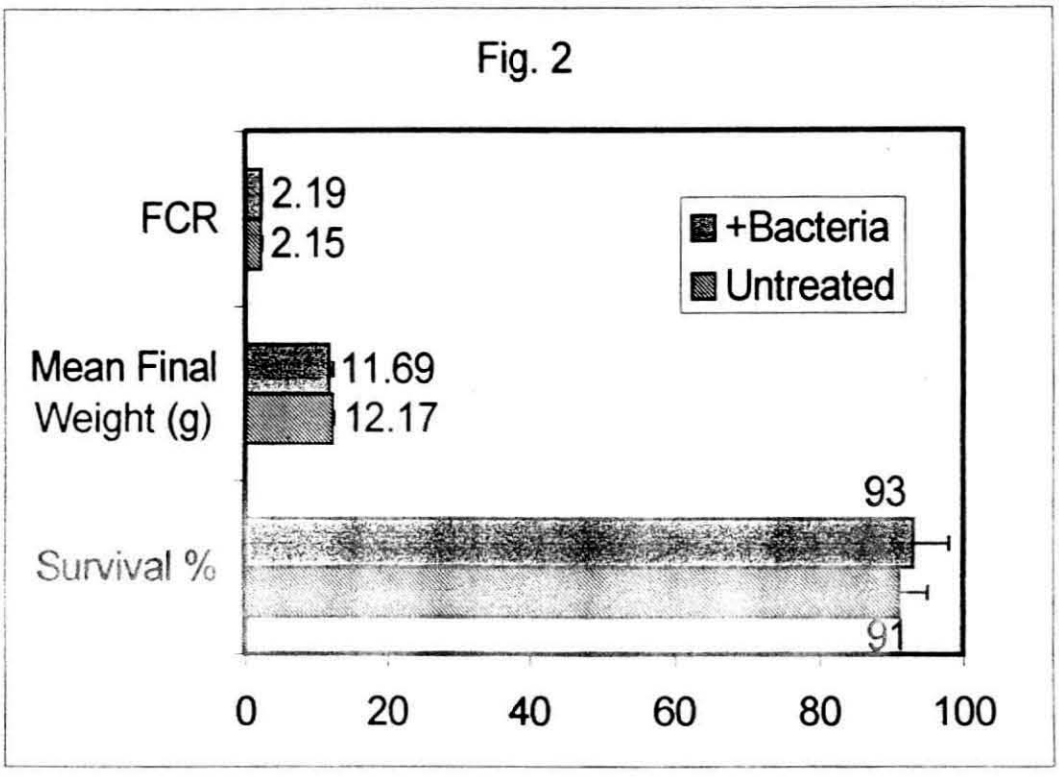
The importance of microbial communities in aquaculture systems and pond productivity cannot be over stressed. Bioremediation agents serve to modify or manipulate the microbial communities in water and sediment such that they reduce or eliminate selected pathogenic microbes and generally improve growth and survival of the targeted species. There are various ways through which bioremediation agents could act in aquaculture systems. These include competitive exclusion of pathogens, enhancing digestion through the supply of essential enzymes, moderating and promoting the direct uptake of dissolved organic materials, active promotion of pathogen inhibiting substances and other possible mechanisms (Jory, 1998). According to Bratwold et al. (1997) the specific ecological applications of microbial ecology management in shrimp ponds include the following: optimising nitrification rates to keep low ammonia concentrations, optimising denitrification rates to eliminate excess nitrogen from ponds as nitrogen gas, maximising carbon mineralisation to carbon dioxide to minimise sludge accumulation, maximising primary productivity that stimulate shrimp production and also secondary crops and maintaining a diverse and stable pond community where undesirable species do not become dominant.

In spite of these theoretical advantages, published results on bioremediation agents, particularly its use in aquaculture ponds, are contradictory. Boyd (1995) and Boyd and Gross (1998) found that bacterial (*Bacillus* sp.) additions in the pond did not improve the water quality as expected. He however observed higher survival of fish in ponds treated frequently with 3 species of live Bacillus. The mode of action was unknown because water quality was not measurably improved. Pond studies also showed that applications of an enzyme preparation tended to enhance microbial mineralisation of organic matter, but no effect on fish production was observed. Boyd and Gross (1998) concluded that too little is known about the modes of action these bioremediation agents, the conditions under which they may be effective, their application rates and methods for general recommendation of their use. Nevertheless, the products are safe to humans and the environment, and their use poses no hazards. Therefore, commercial producers should conduct trails with these products, and researchers should conduct experiments with them.

Only a few properly controlled, well documented and peer reviewed studies on probiotic (bioremediation agents) usage in fish and shrimp rearing have appeared in literature (McIntosh et al., 2000). Most of such studies show that the addition of

probiotics has no effect on the water quality of cultured shrimps (Samocha et al., 1998) and cultured channel catfish (Queiroz and Boyd, 1998). McIntosh et al. (2000) hypothesised that the outcome of microbial supplement addition may not be profound in aquaculture facilities where wastewater is flushed daily out of the system. Hence they conducted a study to evaluate if routine use of a commercially produced bacterial supplement could improve water and sludge quality, and *Litopenaeus vannamei* under zero water discharge with a low protein diet and high stocking density. Fig. 2 shows their results in brief. There was no significant difference in the values of survival, mean final weight and FCR of *L. vannamei* between treated and untreated tanks. Besides, there was no significant differences in water and sludge parameters between the untreated shrimp tanks and those that were treated with the bacterial supplement. This study suggests that producing shrimp with 'zero water discharge' does not have any detrimental effect on the survival, mean final weight, FCR and water quality during the grow-out period. While the application of a bacterial supplement did not damage the animals or water quality, it did nothing to improve these parameters.

Fig. 2. Shrimp survival, mean final weight and FCR of shrimp with or without the addition of a bacterial supplement. There is no statistical difference (*P>0.05*) between treatments. Modified from McIntosh et al. (2000)



There are only few reports (Suhendra et al., 1997; Moriarty, 1998; Prabhu et al., 1999) of bioremediation agents working well in shrimp aquaculture systems. In an experiment conducted in Indonesia, Moriarty (1998) compared luminescent *Vibrio* sp. counts and shrimp production in ponds in which a *Bacillus* sp. based bioremediation agent (PondPro-VC™) was used and those in which it was not used. The value of adding selected strains of *Bacillus* as bioremediation agent to control the Vibrio populations in

farms using the same water sources, which contained luminous *Vibrio* strains, was demonstrated. The farms that did not use the *Bacillus* cultures experienced almost complete failures in all ponds, with luminescent *Vibrio* disease killing shrimps before 80 days of culture were reached. In contrast, a farm using *Bacillus* cultures in abundance (10^4-10^5/ ml) was culturing shrimps for over 160 days without problems. *Vibrio* numbers, especially luminous *Vibrio* numbers were low in treatment pond water and nil in sediment (Table 4). Morairty (1998) concluded that bioremediation agents are a significant management tool in shrimp culture practice, but their efficacy depends on understanding the nature of competition between species or strains of bacteria. Further, they rely on the same concepts that are used successfully in soil bioremediation. Suhendra et al. (1997) also reported similar results on using selected strains of *Bacillus* strains in shrimp ponds in West Java (Indonesia).

Table 4. Total and luminous *Vibrio* counts in pond water and sediment in control ponds and those using *Bacillus* sp. Modified from Moriarty (1998).

Mean values from 6 ponds	**Mean Days of Culture**	**Water (no./ml)**		**Sediment (no./ml)**	
		Vibrio	**Luminous *Vibrio***	***Vibrio***	**Luminous *Vibrio***
Control ponds	45	3300	180	5672	26082
Bacillus treated	79	3224	25	4.15	0

There are approximately 15 species of *Bacillus*, which are the main components of commercial probiotic (bioremediation) products for pond aquaculture (Jory et al., 1998). According to Jory (1998) there are several characteristics that make *Bacillus* an ideal bioremediation agent in aquaculture (see Table 5). The pond environmental conditions must be efficiently managed so that the addition of bioremediation agents can have a significant beneficial effect (Moriarty, 1998).

Table 5. General characteristics of *Bacillus* sp. which make it an ideal microbe for use as bioremediation agent or probiotic. From Jory (1998).

Advantages of using Bacillus sp as Bioremediation Agents

- Bacillus can easily move around (motile) because they have a whip like flagella
- Bacillus form endospores, which are useful under stressful conditions. Endospores allow Bacillus to reproduce when conditions are favourable
- Bacillus produce antibiotics of which bacitracin, polymixin, trycodin, gramicidin and circulin are examples
- Bacillus produce special compounds (enzymes) that can break down

polysaccharides, nucleic acids and lipids

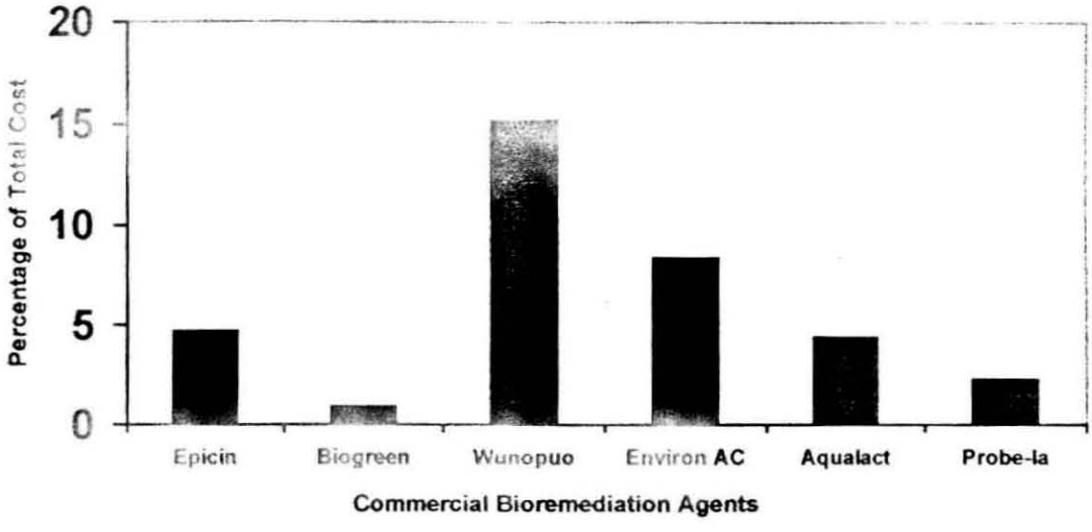
- Bacillus are easily transformable (free DNA is easily incorporated to change its genetic make-up). This is very useful in making 'designer' bacteria
- Bacillus are thermophilic, growing at high temperatures (50-70 °C)
- Bacillus are easy to isolate from soil or air. They grow well on synthetic media. Ammonium can be its sole nitrogen source. Few isolates require vitamin additions

Adequate oxygen levels (e.g., supplemental aeration) are often a key component for bacterial amendments to work efficiently. Decrease in ammonia levels and increase in nutrient levels and total heterotrophic bacterial counts in ponds where *P. monodon* was cultured for 120 days with application of a commercial bioremediation agent (NS Series Super SPO™) was reported by Prabhu et al. (1999). This also resulted in increased daily growth rates and production of shrimp from the treatment ponds.

Although the advantages of the use of bioremediation agents in shrimp farms is still a point of debate, it is clear that applied research in pond microbial ecology can provide important breakthroughs to improve the environmental sustainability of shrimp culture, particularly in view of the recent negative publicity regarding the environmental impact of shrimp farms.

Notwithstanding these controversies, shrimp farmers in India use a wide variety of commercial probiotics (bioremediation agents) during the grow-out period. In a cost analysis conducted recently on shrimp farms in the state of Andhra Pradesh Anikumari et al. (2001) reported that when probiotics are used the cost of production increases by 0.9-15.2%, for an average production of 1.3 t/ha. Out of the 6 probiotics studied, water probiotics (bioremediation agents) after fermentation and feed probiotics showed lower cost per kg production (Fig.3). The production cost is higher

Fig.3. Cost of bioremediation agents as percentage of total cost in shrimp farms in AP (from Anikumari et al., 2001)



for bioremediation agents that are applied directly. In spite of the high costs, farmers are willing to try untested bioremediation agents in their desperation to save their crop. The survey results (Anikumari et al., 2001) show that the farmers are using both water and feed probiotics. Water probiotics are applied either directly or after fermentation and contains multiple strains of bacteria like *Bacillus acidophilus*, *B. subtilis*, *B. licheniformis*, *Nitrobacter* sp., *Nitrosomonas* sp., *Pseudomonas* sp., *Alcaligenes* sp., *Lactobacillus* sp., *Cellulomonas* sp., *Aerobacter* sp., and the yeast *Saccharomyces cerevisiae*. Testing these commercial bioremediation agents for its cost effectiveness in local farm conditions and setting out the correct protocol for its use is imperative before such products are marketed.

Probiotics in live feeds – Bio-encapsulation

Marine larval rearing involves feeding the hatched larvae with suitable live feeds (diatoms, rotifers, copepods, nematodes, *Artemia* nauplii and metanauplii, mysids etc). Most often live feeds are the primary source of bacterial contamination in rearing systems. By virtue of their size and feeding habits, most live feeds are size specific filter feeders. Therefore, it is possible to incorporate into the live feed particles (say an antibiotic or therapeutic drug like Romet-30 or a probiotic organism) of the appropriate size. This process called bio-encapsulation is thus an innovative means of delivering drugs and probiotic organisms to the larvae (Mohamed, 2001). Indeed, for fish larvae that are active sight feeders, it is the only effective means of drug delivery and several studies have been made on this aspect.

Enrichment of *Artemia* nauplii with a known probiotic yeast *Saccharomyces boulardii* and its role in enhancing resistance against the pathogen *Vibrio harveyi* was investigated by Patra and Mohamed (2003). *S. boulardii* (SB) was cultured, then fed to Instar II *Artemia* nauplii in three different treatments; 10^2 (T1), 10^3 (T2) and 10^4 (T3) colony forming units (CFU) per ml in triplicate. The algae *Nanochloropsis* sp. was used as control diet. Survival and total count of CFU nauplii $^{-1}$ was observed on different media (Sabouraud, for enumerating yeasts, Thiosulphate Citrate Bile salts Sucrose, for enumerating *Vibrio* and Seawater Agar, for enumerating total aerobic flora) for each replication. Enhanced survival of nauplii was observed in treatments as compared to control. Results indicated that enrichment of *S. boulardii* in *Artemia* nauplii proceeded in a linear fashion, and up to 3500 CFU of *S. boulardii* could be detected in one nauplii at 10^4 CFU ml^{-1} treatment. No conclusive trend could be observed in the count of *Vibrio* and total aerobic flora due to treatment. Enriched nauplii were then challenged with the pathogen *V. harveyi* for 24 h and 48 h at a concentration of 6.1 x 10^6 CFU ml^{-1}. The survival counts at 48 h showed that the resistance of the nauplii was significantly ($P< 0.01$) improved in those fed with 10^4 CFU ml^{-1} *S. boulardii* (90% survival rate after 48 h of challenge versus less than 40% for the infected control group without SB and treatments T1 and T2). This study shows that *S. boulardii*, which has been used for the first time in an aquatic live feed organism, has a profound beneficial effect on the nauplii by increasing its resistance to a pathogenic *Vibrio* infection.

Microalgal cultures are a virtual storehouse of various microorganisms, and therefore, by feeding marine microalgae to marine larvae, we transfer many potentially pathogenic microorganisms to the culture medium. The consequences are low survival and poor quality larvae, besides failure of microalgal culture due to over-growth of microorganisms. Probiotic organisms have been incorporated into microalgal cultures with remarkable benefits. In a recent study Rajiv (2003) found that the the addition of the probiotic yeast *S. boulardii* as a single addition to *Chaetoceros* culture resulted in significantly ($P < 0.01$) improved (162% increase in maximum algal density) algal growth rates with prolonged stationary period when compared to the control. It also helped in keeping the total aerobic bacterial counts and total Vibrio counts on TCBS in the medium to very low levels.

Perspectives and Conclusions

It is well known that microorganisms cannot be avoided in aquaculture operations (Ringo and Birbeck, 1999). The key to successful management of aquaculture operations lies in the manipulation of these microbes through innovative means such as use of probiotics. The state of the art of aquatic probiotics has not reached to the level found in land animals. The application of probiotics for fish and shrimps, either as a biocontrol or as a bioremedial measure shows promise, but much more research efforts are needed to come to a complete understanding. Gatesoupe (1999) stated that the first question that remains unanswered in most cases is the fate of the probiotic organism in the rearing medium or in the gut. More investigations using molecular and immunological approaches may yield better results.

Even without much research backing, a vast number of commercial probiotic products are being used by shrimp farmers, mostly under pressure from marketing agents and peers. It is essential that proper testing of these products under local environmental conditions be done before they are marketed. Government research laboratories therefore, have to equip themselves for carrying out tests of these products and ascertain the factual in the claims. At the same time, the search for new and better probiotics should continue. Unlike endothermic animals, the ubiquitous environmental microbe *Vibrio* dominates the gut microflora of fishes and shrimps. It is very likely that non-pathogenic Vibrios hold the key to isolating and developing a successful probiont for use in aquaculture.

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TECHNIQUES FOR QUALITY PEARL PRODUCTION

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Introduction

The success of pearl production adopting culture techniques depend on the quality of pearls rather than the quantity produced. It is quiet evident that the cost of production would go up in future and hence pearl quality alone can meet their challenges to make profits in this industry. There is long way to go to meet the challenges through application of advanced biotechnological tools for quality pearl production. The techniques related to pearl production being a biological process, priority for production of quality pearls can be achieved mainly by improvement in the quality of mother oyster stock and also by manipulation of environmental characters and through tissue culture.

Quality improvement of mother oyster stock

At global level the pearl culture mainly depend on the wild stock for pearl production and when the pearl oyster population in the natural beds shows a sign of depletion, the techniques of spat collection from the wild have been adopted. During this period the genetic qualities of wild stocks are not ascertained. It is feared that the limited stock may be degenerating in terms of pearl quality. Hence there is an urgency to identify the genetic characteristics of wild stock and to build up the selected stock capable of producing desirable quality of pearls, resistant to diseases, adaptability to environmental stress, high growth potential. In Japan such studies were already taken up in *P. fucata.* Through such studies Wada (1975, 1984, and 1986) demonstrated that pearls without yellow pigments could be produced. Hatchery technology has been developed in several countries for the commercially important species *Pinctada maxima*, *P.margaritifera* and *P.fucata*. A selective breeding programme can easily be carried out to develop a particular strait of character to be inherited. In India selective breeding programme has already been initiated in *P. fucata* to improve the colour and quality of pearls and also to increase the shell cavity to accommodate large sized nucleus. These characters have been found to inherit in *P. fucata.*

In India the consecutive use of the succeeding generations as parent stock resulted in the poor growth of the successive generations hence it was suggested to out breed those stocks. The inbreeding of these stocks resulted in the production of higher percentage of yellow nacre, and more thickness of nacre was also observed. Hence from the natural stocks a breeding stock is selected based on the desired characters and the next generations produced by assortative mating of the selected group. Genetic profiling of proteins from the selected oysters of wild stock as well as the newly produced generations reveals the heritability of the characters. By repeating the selection and genetic marking of proteins of the heritable characters we can produce a better stock of oysters, which produce better quality pearls

Pearl culture environment

The quality of pearl is influenced by the environmental factors. Basic studies on the pearl culture grounds have been made in Japan on this aspect. The outcome of the study facilitated the establishment of farms at suitable sites and shifting of culture grounds for improvement of quality of pearls. Availability and quality of phytoplankton, which form the food item of pearl oysters, influence pearl quality (Matsui, 1960) via its role of energy supply. The type of phytoplankton available at different seasons at different sites has been studied thoroughly. Studies revealed that phytoplankton's influence the colour of pearls and areas were identified and the pearl bearing oysters were shifted to the site before pearl harvest in order to get a final coating of desirable colour. This is otherwise called 'make-up' culture. Similarly the amount of trace elements influences the colour of pearls. The level of these trace elements at different pearl culture grounds has been studied thoroughly in Japan. The locality having optimal level of trace elements was chosen for rearing nucleated oysters. At Tuticorin, preliminary trials indicated that it is possible to manipulate the colour of the nacre and thence the pearl in captivity. The medium lethal concentrations (LC 50) of Copper, Cobalt and Ferric were administered through feed and initial results show the differentiation in colour of new nacre secreted with bluish nacres by Cobalt below LC50 concentrations.

Influence of depth on quality pearl production

Apart from the environmental factors such as temperature, salinity, pH, turbidity, detritus, plankton, trace elements, nutrient salts, the physical factors such as the depth, light intensity, topography of area, wind velocity, tidal effect and wave action play a composite role in pearl quality. Among the characters; depth plays prominent role in pearl quality and the temperature varies with the depth. Culture practices of oysters are decided on the optimal level of temperature. For example, in summer the surface temperature in the Ago Bay, Japan was around 28 to 30°C and hence the nets were lowered to a depth of about 7 m where the temperature was around 23 to 26 ° C. During spring and autumn the surface water temperature was around 23 to 25° C and therefore the nets are kept at 2 to 3 m depth (Alagarswami, 1970).

Dharmaraj (2002) studied the influence of depth on pearl quality. The results indicated that the pearl growth was 12 % more at 1 m depth than in 3 m depth. In view of this, the cages containing nucleated oysters in Indonesia are kept at the seabed having a depth of 10-20 m in order to get good quality pearls (Tun and Winanto, 1988). When the cages are suspended at 5-8 m depth during dry season they are subjected to strong sunlight, which induced nacre-secreting cells to produce calcite crystals resulting poor quality pearls. The lustre and colour are better at higher depths although the rate of nacre deposition is slow.

The pearls from 1 m depth were of ivory in colour having poor lustre whereas the pearls from 5 m depth were of light yellow in colour with good lustre. Knowing the influence

of depth on pearl quality, the pearl farms in Australia are located at 33 m depth and in Japan at 10 m depth (Alagarswami, 1970).

Improvement in surgical techniques

The pearl quality could be improved by treating the graft tissue with appropriate stains, which are capable of activating the nacre secreting cells and by taking utmost care in the surgery procedures. The application of stains like azumin and eosin was to improve the quality of pearl, percentage pearl production and survival of operated oysters. When the implantation is made with graft tissue treated with azumin, the oysters showed high survival when compared to eosin or filtered seawater treated ones. The treatment with stains (azumin and eosin) improved the pearl production in *Pinctada fucata*.

Several other surgical techniques were also employed on he improvement of pearl quality, retention of nucleus, prevention of contamination, enhancement of pearl production, reduction of oyster mortality, improvement in wound healing process. One of such techniques in surgical improvement was the use of tetracycline hydrochloride (2 % TC-HCL), 0.4 % succinated antherocollagen and polyethylene glycol 6000 coating on the nucleus. The survival rate of oysters treated with this antibiotic was higher (86.7 %) than that of the control group (63.3 %) treated with uncoated nuclei. The nuclei retention rate was also high in the treated group.

Norton *et al* (1976) and Aquilina (1999) carried out a research programme aiming at improving the production of gem quality pearls in *P.margaritifera*. Treatments incorporating modern surgical techniques were applied to seeding operations. Use of prophylene phenoxetol for narcotization of oysters and the use of cyanocrylate adhesives to fuse incision site yielded poor results. But efforts are being undertaken using modern techniques.

The Pearl Developing Group (PDG) carried out improvement of quality of pearls with PDG alpha antibiotic coating on nucleus. PDG is able to offer three distinct advantages to pearl farmer for better price, better quality and better science.

Biotechnological aspects are undertaken in Japan in which the nuclei are coated with antibiotic powder. It is inevitable that insertion of nucleus after causes infection in oysters. It resulted either in the rejection of nucleus or the death of oysters. Therefore the nucleus coated with antibiotic powder was to improve the retention rate of nucleus from 70 to 80 % as against 50 to 60 % with uncoated nuclei.

Studies on bio – mineralization of nacre

Elaborate work on mineralisation and spectral characteristics of pearls was undertaken (Wada, 1972, 1983). The outer epithelium of the mantle of bivalve molluscs secretes extrapallial fluid that crystallizes in to aragonite crystals. The fluid contains carbonate and other inorganic ions (Wada, 1972). The organic matrix and the growth of nacreous layer are formed alternatively. The nature of crystals either aragonite or calcite and their size are related to the secretary activity of the outer mantle epithelial cells. The

activity differs in species, in environment and in physiological condition of animal (Wada, 1972). A thorough knowledge on these aspects would help in controlling the quality of pearl. These studies may have to be taken up in future for improved quality pearl production.

Tissue culture techniques

Culture of mantle tissue of pearl producing molluscs has been undertaken in recent years. Machii (1974) cultured mantle tissue of *P. fucata* and reported the types of cells proliferated from the tissue and the secretion of organic substance in *in-vitro*. The latest breakthrough obtained in the culture of mantle tissue of *P. fucata* and the abalone *Haliotis varia* is a milestone in tissue culture research. It created the possibilities of not only the production of pearls in large numbers but also different coloured pearls.

In the context of the dwindling quality pearl production initiated mantle tissue culture of the Indian pearl oyster, *Pinctada fucata* and the progastropod abalone *Haliotes varia* was organized in the laboratory. In the explant tissue culture, the cells proliferated and migrated away from the explant and multiplied *in-vitro*. This resulted in the formation of a cell sheet. The round cells develop pseudopodia that later covered the entire surface of culture plates and formed an organic matrix.

In an organ culture, the mantle tissue of a pearl oyster/ a kept in nutrient rich medium resulted in the formation of nacreous layer with organic matrix and a pearl sac within 3 months after organization of cultures. The basic technology developed through tissue culture method can totally eliminates the dependence on natural environment for pearl production. It provides scope for manipulation of the technique to produce pearls of the desired quality.

By organizing explant cultures of pearl producing molluscs, the epithelial cells capable of producing aragonite crystals may be collected and stored in cell bank. The cells can be used at any time for the production quality pearls in *in-vitro*. The cells in suspension would form the pearl sac that would secrete nacre to form a pearl. Isolation and the type of epithelial cells that would secrete the aragonite crystals, which form the top quality pearls, can be done.

MARINE INVERTEBRATE TISSUE CULTURE TECHNIQUES AND ITS APPLICATION IN PEARL PRODUCTION

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Introduction

Tissue culture, in general, is being carried out in various fields of medical and agricultural research. The study has been commenced since long back to understand the cell type, cell behaviour, cell structure, cell multiplication, cell reaction to drugs etc. It has become a vital tool in micro pathological and immunological studies aiming at finding solutions to certain diseases. Plant tissue culture has reached an advanced stage of achieving an entire plant from a single cell. All these studies are concerned with plants / animals which are related to freshwater species. Marine invertebrate tissue culture is not only a new origin but also a new field of research concerned with marine animals. Primary aim of the study is to formulate suitable culture media specific to each species and to establish cell lines. Later the study is extended to commercial aspects of producing *in-vitro* pearl from pearl oysters in Japan. Extensive works have been carried out on cell proliferation and its behaviour in a medium developed specifically for the species and to formulate suitable medium based on the results obtained. The countries like Japan, China, United States and Canada initiated marine invertebrate tissue culture. Among these countries, Japan is the pioneer country carrying out research in pearl oyster for the purpose producing *in-vitro* pearl through tissue culture. Visualizing the importance of the work in view of deterioration of natural environment India too entered in to the field of marine invertebrate tissue culture research, as it is one of the pearl producing countries in the world. Expertise in the field of research has already been developed and a fully functional marine invertebrate tissue culture laboratory has been established at Tuticorin for the first time in India. Cultures are organized since 1996.

Set up of tissue culture laboratory

Generally the tissue culture laboratory should be compact with different modules so as to contain contamination by effectively maintaining high-grade hygienic conditions. It is fully air-conditioned. The entrance room is the one where the records are maintained and discussions are held prior to organization of cultures. Animal sterilization room is arranged on the left side of entrance room having U.V. sterilization unit and provisions for running seawater supply. The entrance room leads to preparation room where preparation of culture media, saline solutions, extracts, tissue culture materials etc., are carried out. The preparation room proceeds to dressing room and to operation room or clean room. A dark chamber or otherwise called 'Pass Box' is situated in between the preparation room, dressing room and clean room. It has three doors with a U.V.light on its top to keep the materials always sterile. The doors are arranged in such a way that one door is facing

preparation room through which sterile materials are placed inside, the other on the dressing room from where the dress materials are taken out and the third one on the operation room from where the materials are taken during organization of cultures.

Preparation of culture media and balanced salt solution

The marine mollusc medium (MMM) is constituted based on the composition of haemolymph of each species. Refinement of medium is done periodically based on the results obtained in the cell culture. There are a few media developed for marine mollusc. They are Medium 199, P35, L-15 and Ham's F 12. The media are commercially available. They can either be prepared by following the formula.

The balanced salt solution (BSS) is prepared in the following manner.

Na. K. solution	125 ml
Mg. Solution	50 ml
Triple distilled water	200 ml
These are mixed, autoclaved and taken to clean room	
Ca. solution	50 ml
Triple distilled water	50 ml

These two solutions are mixed, autoclaved and taken to clean room. Mixing of the above two solutions and the following is done on the clean bench.

Glucose	5 ml
$NaHCO_3$	5 ml
NaH_2PO_4	5 ml
Kanamycin	0.5 ml
Penicillin	5 ml
Fungizon	5 ml
Total	500 ml

Preparation of animals and tissues

The test animals are depurated for a minimum period of 3 days in U.V.treated running seawater. The depurated animals are wiped with externally with 70 % alcohol and taken to clean room. The mantle tissues of test animals are excised and washed in BSS to get rid of mucus and other adhering particles. The tissues are cut in to tiny pieces of 1 square mm in size.

Culture techniques

Flask and Petri dish cultures: Before introducing the fragments of tissue in to the culture flask the mouth of the flask is shown to isopropanol flame for sterilization. Tissues are placed inside the flask with the help of a needle. The tissues are allowed to stick on to the flask and 3 ml of medium is added. A similar inoculation is made in petri dishes also. The culture plates are placed in CO_2 incubator and maintained at 25-28°C.

Cell well culture: The cell well is otherwise called as micro plates. There are different types of cell wells. The size of 24 wells is 16 mm in diameter and 17 mm in height and the size of 96 wells is 6.4 mm diameter and 11 mm height. The cell well is provided with a cover. The cell well is used to culture single cell for the purpose of cloning. 3 to 4 drops of medium are added to each well. The cell wells are kept in CO_2 incubator at 25-28°C.

Medium change: Medium change is normally done on alternative days. Periodicity of medium change is determined by observing the condition of the cultures. Culture flasks are taken to clean bench after wiping with 70 % alcohol. When the flask is opened, it is shown to flame. Much care is taken during medium change. A separate pipette is used for each flask. Half of the medium is changed during first and second time and subsequently the whole medium is changed. At times cell suspension is centrifuged and fresh inoculations are made. In some established cell lines the cells are active and hence the entire medium is changed.

Organization of cultures

1. **Primary culture:** The processed tissue is treated with trypsin for the purpose releasing the cells from the tissue. To effect this the cut pieces of tissues are placed in trypsinisation flask containing 30 ml on marine mollusc calcium magnesium free phosphate buffer solution (MM CMF PBS) with 0.05 % trypsin. A teflon stirrer is used in the flask for proper dissociation of tissues and dispersion of cells. The stirring is done for 10-15 minutes at 1200 rpm. The cell suspension is first filtered through 150 µm sieve and then through 60 µm sieve. The filtrate is centrifuged at 4°C for 5 minutes at 800 rpm and the supernanent solution is removed gently without disturbing the precipitate. A drop of medium is added to the precipitate and mixed well. The mixture containing free cells is distributed to different flasks or petri dishes by means of Pasteur's pipette. 3 ml of medium is added to each flask and the flasks are placed in CO_2 incubator at 25-28°C.

2. **Explant culture:** For explant culture of tissues, fragments of tissues are processed in balanced salt solution (BSS) and inoculated in the flasks or petri dishes. 3 ml of medium is added to each flask. The cells from the explant proliferate in large numbers and migrate away by adhering to the bottom of the flask. The round epithelial-like cells and fibroblast-like cells are seen in the cultures. The cells do multiply in *in-vitro* cultures and increase in numbers forming cell sheet. When a cell sheet is fully formed, it is due for subcultures or for cryopreservation of cells. At ideal conditions the cells develop pseudopodia and form a network to cover the entire surface of the flask as organic matrix. The migrated cells are stationed at places and formed pearl sac. The organic matrix induces the cells to secrete crystals.

3. **Organ culture:** The processed fragments of tissues are placed on a raft in petri dishes. The raft may be at any form as per the requirement of the experiment. In organ culture the explant tissue is not immersed in the medium but it is kept in such a way that the medium is filled up to the lower phase of the tissue leaving the upper phase with air contact. In such case the cells are kept intact without dislodging their

positions. The interaction and integration of the cells perform their original functions of forming organic matrix and pearl sac. The cells secrete nacreous crystals and deposit on the matrix. As the mantle cells are responsible for the formation of shell, the cells secrete prismatic layer in hexagonal form. Each hexagonal segment is bordered by interlamellar organic matrix.

Preservation of cells

Cells to be preserved by freezing would be released from the culture flask by adding 0.25 % of trypsin. The cell suspension with 3 to 6 ml of medium is centrifuged for five minutes at 1200 rpm at 4°C. Supernanent water is removed and 2 ml of medium and 2 ml of Minimum Essential Medium (MEM) with Dimethyl Sulfoxide (DMSO) 7.5 % mixture were added drop by drop. The 4 ml suspension is divided into four parts and kept in four freezing vials. After the vials are sealed and labeled, there are frozen at the rate of -1°C at every minute. Freezing is done at three stages, first at 0°C for 30 minutes, then at -20°C for 60 minutes and thirdly at -70°C for 6 months and finally at- 196°C for one or two years in liquid nitrogen.

In order to protect from damages of cells during storage, DMSO 7.5 % and glycerine 10 % are used along with medium. Freezing of cells is done mainly for three reasons.

1. During cell line the cells may change their enzyme activity, chromosome number etc. Therefore it is essential to freeze these cells at a particular stage of cell line and then rejuvenated.
2. There may be contamination in cell line. To prevent this cells are frozen at periodic intervals.
3. In an established cell line the cells can be cultured to a maximum of 50 times. In some other cell line, cells are likely to die at any time. Such cell lines can be sub-cultured only for 30 times. Freezing of these cells may extend the period of cell line.

Application of tissue culture techniques

There is an increasing use of tissue culture in various fields of biological research. Tissue culture techniques are being adopted in Marine Invertebrates since in recent years. By conducting tissue culture, valuable information could be collected on aspects of like cell structure, cell division, cytogenetics, cell physiology and cell viability. Tissue culture techniques are useful in studying the structural as well as functional aspects of cells, tissues or organs by culturing them *in-vitro*. The techniques are employed in investigating the effect of chemicals and radioactive elements on normal tissues and cancer cells and in microbiology, pathology and in the production of vaccines. Results obtained may help in finding out methods of curing several diseases. Careful studies in tissue culture will help in transplantation of tissues and cells among members of a species or from one species to another species. In recent years tissue culture technique is being used in the production of *in-vitro* pearls from pearl producing molluscs.

HYBRIDOMA TECHNIQUE AND MONOCLONAL ANTIBODIES FOR MARICULTURE APPLICATIONS

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Introduction

Ever since the beginnings of experimental immunology at the end of the nineteenth century, scientists have exploited the specificity of antibodies to detect, isolate and analyze biological material. The power of antibodies as probes for biological structure underwent a quantum increase in 1975, when Kohler and Milstein developed the technique for production of Hybridomas. Hybridomas are immortal somatic cell hybrids that secrete Monoclonal antibodies (MAbs) of predefined specificity. These antibody-secreting cell lines can be established routinely and maintained *in vitro.* By combining the nuclei of normal antibody-forming cells with those of their malignant counterparts, Kohler and Milstein developed a powerful way of analyzing and purifying individual molecules within the enormously complex mixtures in biological material.

In order to understand the revolutionary impact of monoclonal antibodies, it is necessary to understand the problems and limitations of conventional serology. While the specificity of antibodies provided a way of overcoming the enormous complexity of biological material, the production of highly specific antisera was difficult and unreliable. It required highly purified antigen. However, using hybridoma technology it is now possible to produce unlimited quantities of exquisitely specific antibodies against virtually any molecule, regardless of the purity of the immunizing antigen. The fine specificity, degree of cross-reaction, affinity and physical properties of antibodies may be selected to suit individual needs.

Conventionally, polyclonal antibodies are prepared in rabbits for use in diagnostics, serotyping and vaccine development. Rabbit antiserum contains many different types of antibodies derived from several plasma cell clones that are specific to different epitopes. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques such as background reaction and false positives. The availability of antiserum in limited quantities is also a serious drawback. Therefore the preparation of large quantity of homogenous antibodies with a defined specificity was a long-standing goal of immunochemical research. This was achieved with the development of hybridoma technology for production of MAbs.

Production of Hybridoma

In animals, antibodies are synthesized primarily by plasma cells, which are terminally differentiated B Lymphocytes. Because plasma cells cannot be grown in tissue culture, they cannot be used as an *in vitro* source of antibodies. For decades, immunologists have sought ways of producing homogenous antibodies of defined specificity. Hybridoma technology allows the growth of clonal populations of cells secreting antibodies with a defined specificity. Here, antibody-secreting cells isolated from an immunized animal is fused with myeloma cell, a type of B cell tumor from BALB/c mice. These hybrid cells or hybridoma can be cloned and maintained *in vitro* to secrete antibodies with a defined specificity, which are known as monoclonal antibodies.

The usefulness of monoclonal antibodies stems from three characteristics – specificity of binding, homogeneity and ability to be produced in unlimited quantities. Another unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single cell cloned prior to use, monospecific antibodies can be produced after immunizations with complex mixtures of antigens. However, it would be wrong to think that monoclonal antibodies will completely replace conventional serology. The production of monoclonal antibodies involves a great deal of work, and a high level of commitment. There will often be occasions when the effort required may not be justified. They are not the best choice for certain immunochemical techniques. In theory, either as single antibody preparations or as pools, monoclonal antibodies can be used for all of the tasks that require or benefit from the use of polyclonal antibodies. In practice, however, producing exactly the right set of monoclonal antibodies is often a difficult and laborious job.

In a hybridoma, the myeloma cells provide the correct genes for continued cell division in tissue culture, and the antibody secreting cells provide the functional immunoglobulin genes. Hybridomas can be prepared by fusing myelomas and antibody secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used. Therefore fusions are normally done with cells from the same species. All commonly used mouse strains can serve as successful fusion partners with BALB/c myelomas, however immunizations are normally done in BALB/c mice.

Polyethylene glycol (PEG) is the most commonly used agent to fuse cells in hybridoma production. PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells. Because of the abnormal number of chromosomes, segregation does not always deliver identical sets or chromosomes to daughter cells, and chromosomes may be lost. If one of the chromosomes that carries a functional, rearranged immunoglobulin heavy or

light chain gene is lost, production of antibody will stop. In a culture of hybridoma cells, this will be seen phenotypically as a decrease in antibody titer and will result in unstable lines. If the chromosome that is lost contains a gene used in drug selection, then the growth of the hybridoma will be unstable, and cells will continue to die during selection.

Drug selection for elimination of unfused myeloma cells

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused, and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in culture. The cells from the immunized animal (antibody secreting cell) do not continue to grow in tissue culture and so do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed, which can be achieved by drug selection. Commonly, the myeloma partner has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis. For eg. Selection with 8- azaguanine often yields a cell line harbouring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). The addition of any compound that blocks the *de novo* nucleotide synthesis pathway will force cells to use the salvage pathway. Cells containing a non-functional HPRT protein will die in these conditions. Hybrids between myelomas with a nonfunctional HPRT and cells with a functional HPRT will be able to grow. Selections are commonly done with aminopterin, methotrexate or azaserine.

Stages of Hybridoma Production

It is convenient to divide the production of monoclonal antibodies into three stages: (1) immunizing mice, (2) developing the screening procedure, and (3) producing hybridomas.

Animals are injected with an antigen preparation, and once a good humoral response has appeared in the immunized animal, an appropriate screening procedure is developed. The sera from test bleeds are used to develop and validate the screening procedure. After an appropriate screen has been established, the actual production of the hybridomas can begin. Several days prior to the fusion, animals are boosted with a sample of the antigen. For the fusion antibody secreting cells are prepared from the immunized animal, mixed with myeloma cells and fused. After the fusion, cells are diluted in selective medium and plated in multiwell tissue culture dishes. Hybridomas are ready to test beginning about 1 week after the fusion. Cells from positive well are grown and then single-cell cloned. Single-cell cloning ensures that cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained.

Characterization of Clones

The total characterization of a MAb is a long and complex procedure, which varies widely with the intended use of the antibody. If a single hybridoma has been produced and is intended for a specific function it is unlikely that the antibody produced will have all the required characteristics. To produce MAb superior to conventional sera for most highly specialized functions it is better to make a large panel of cloned hybridomas and further select from these by characterization of the antibodies they produce.

Antibody produced by a clone belongs to one single class, and hence antibody class indicates number of clones in a population. Antibody class is most readily determined by the use of class specific antibodies in an ELISA or by Ouchtherlony assay. Determination of antibody class does not constitute final proof that an antibody is monoclonal. For some of the more sophisticated uses of MAbs this is essential. While it is likely that there is only one cell type if the antibody produced can be shown to be only of the IgM class, it remains possible that there are two or more IgM-secreting hybridomas in the culture. An isoelectric focusing gel is the only final method of proof.

Storage of Hybridoma

Hybridoma and myeloma cell lines can be stored at -80°C or liquid nitrogen by slowly freezing cells in an appropriate solution of nutrients and a cryoprotectant such as dimethylsulphoxide (DMSO). Freezing of cells, which have not been cloned after the primary fusion is not always successful, presumably because of overgrowth by non-producing cells. However, the parent myeloma and established hybridomas can be stored with little difficulty. The cells are centrifuged and resuspended at 5-10 X 10^6 cells/ml in medium containing FCS and DMSO. Aliquots of this suspension are then pipetted into plastic storage ampoules and frozen slowly at -70°C for at least 12 h and then stored at -80°C or transferred to liquid nitrogen

Production of MAbs

MAb production *in vitro*

Mabs can be produced *in vitro* and *in vivo.* For production *in vitro,* hybridomas are best expanded slowly by transfer to 24 well tissue culture plates followed by 25 cm^2 flask and a 75 cm^2 flask containing suitable medium such as Dulbecco's Modification of Eagles Medium, DMEM or Rosewell Park Memorial Institute (RPMI) medium, containing sera (fetal calf serum, FCS), antibiotics and other required chemicals. The cell density is maintained between 10^5 and 10^6 cells/ml. Typical culture supernatants yield up to 100µg/ml of antibody, the exact amount depending upon the cell density and rate of growth. Culture *in vitro* provides a more pure preparation of antibody. The only contaminants are from the FCS. Contamination may be further reduced by the use of serum-free medium.

MAb production *in vivo*

For producing MAbs in *vivo,* mice are primed by intraperitoneal injection of 0.5 ml pristine (tetramethyl pentadecane) 5 – 10 days before intraperetoneal inoculation with 10^5 - 10^7 hybridoma cells. It is important to use mice, which are histocompatable with the parent cells. The rate of growth of the resulting ascites tumour is in general very variable and can be from less than two or more than five weeks. The ascites fluid can be collected from an anaesthetized mouse. It is possible to obtain 10 ml of ascites fluid or more from a mouse by regular tapping. Ascites cells recover from freezing exceptionally well and can be frozen down in the same way as tissue culture cells and reintroduced into animals without difficulty. Ascites fluid will be contaminated with mouse imunoglobulins to a small extent and if a very pure antibody is required this may prove inconvenient.

Purification and storage of MAbs

In many cases, purification of antibody is not necessary since all that is required is specificity and each batch of antibody can be tested for titre and used directly. However, if a MAb is required as a standard reagent or for therapeutic purposes it must obviously be purified. In addition, many of the methods used to characterize a MAb involve labelling it with either enzymes or isotopes and a pure antibody is obviously more suitable for this purpose. Before purification it is important to determine the class of antibody. As with all protein-purification techniques, antibodies may be separated according to charge or size. In addition, they may be purified by affinity chromatography utilizing protein A for certain types and species, by anti-immunoglobulin columns, or by antigen affinity columns if large amounts of antigen are available.

Most MAbs are stable and can be frozen and thawed readily. It is, however, advisable to check the titre after freezing and thawing at an early stage. Many IgM antibodies do not retain full antigen-binding capacity after freezing and thawing but many do. In general, an antibody, which does not freeze well, is better discarded unless it is of considerable importance in which case it should be stored in 0.1% sodium azide at 4°C. MAbs like serum should not be repeatedly frozen and thawed and it is best to freeze 4 or 5 small aliquots from each tissue culture flask or ascites fluid, from the bulk for general use or for testing before purification on large scale.

Applications of Monoclonal Antibodies in Mariculture

Hybridoma technology for production of monoclonal antibodies has contributed significantly to aquaculture. Monoclonal antibodies are being employed in disease diagnosis, pathogen classification, epidemiological analysis and development of vaccines.

Outbreak of disease problems of the cultured fish/shellfish is a major bottleneck faced by aquaculturists. Generally we go for routine diagnostic procedures like microbiological examination and histopathology, which are time consuming. Development

of rapid immunodiagnostic techniques based on polyclonal antisera is faced by problems of cross-reactions and inconsistency in results. If MAb based diagnostic kits (which are more specific) are available for important microbial pathogens, it will help us in adopting more scientific health management measures. MAbs can be used to confirm the presence of pathogens fixed in tissue sections or tissue imprints by immunohistochemistry, particularly useful for detecting low-level infections, which would be overlooked by traditional methods. MAb based immunodiagnostic kits such as ELISA (Enzyme Linked Immunosrbant Assay) and Immunodot can be simplified to the field level for use by farmers. Monoclonal antibodies to several viral and bacterial pathogens of fish and shellfish have been developed. It has been possible to develop rapid, simple, cheap, specific and sensitive MAb based immunodiagnostic kits for several microbial pathogens. In India, College of Fisheries, Mangalore has developed MAbs to *Aeromonas hydrophila*, EUS fungus *Aphanomyces invadens* and white spot virus of shrimp.

Furthermore, detection of minute serological difference among bacterial and viral variants of fish and shellfish is possible by MAb based epitope analysis. This has helped immensely in serological and epidemiological studies. Monoclonal antibodies have also been used in detection of epitopes involved in pathogenesis for development of subunit vaccines.

A serious bottleneck in developing standardized diagnostic assays for most important fish diseases is the lack of antifish immunoglobulin reagents. Hence there is also immense scope for developing Anti-fish immunoglobulin monoclonals using hybridoma technology.

CRYOPRESERVATION OF MARINE FISH GAMETES FOR MARICULTURE APPLICATIONS

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Introduction

Gamete cryopreservation is a wide and complex topic. It is not confined to Cryobiology alone, but also encompasses into complementary disciplines like Reproductive Biology, Cell Physiology and so on. Cryopreservation of gametes also covers invertebrate and vertebrate species and it addresses various cell and tissue types, more complex sperm aggregates called spermatophores and multifunctional tissues like eggs and embryos. While cryopreservation of eggs and embryos is still at stage of basic research, spermatozoa can be successfully cryopreserved for most species and in particular for several teleost fish, the method has already reached a standard that makes it interesting for routine and commercial use.

The importance of cryopreservation steadily increases for purposes of fish culture and conservation of biodiversity. Compared to fresh water species, a high percentage of marine fish spermatozoa survive cryopreservation, as a consequence, the success in breeding is greatly enhanced by the use frozen sperm. Because of the simplicity of the technique, cryopreservation of sperm in marine species is suited for large scale application in Mariculture. As marine fish farming expands, there is an increasing need to apply sperm cryopreservation techniques.

The benefits of this technique include: (1) synchronization of gamete availability of both sexes, (2) use of total volume of milt available, (3) simplicity of broodstock maintenance, (4) transport of gamete, (5) avoiding aging of the sperm,. (6) experimental programmes, (7) conserving genetic variability and (8) protection of endangered species. In protogynous hermaphrodite species, such as black grouper *Epinephelus malabaricus,* sperm can only be collected in inverted males 5-10 years old and success in breeding is greatly enhanced by the use of cryopreserved spermatozoa. Of particular interest to marine biotechnologists is in genetic studies, development of hybrids, clones, polyploids and disease resistant strains.

Principles of cryopreservation

Around 0°C, spermatozoa can be stored for few hours to several days, while cryopreserved gametes can theoretically be stored indefinitely without much deleterious effect. Storage temperatures below −130°C, all biological molecules become motionless and therefore, cannot participate in any biochemical reaction and hence the metabolic

activity is arrested. A commonly used storage temperature is −196°C (temp. of liquid nitrogen) simply because liquid nitrogen is a convenient and widely used storage medium.

The freezing and thawing of biological material involves a series of complex and dynamic physico-chemical process of heat, water and ionic transport between cells and their surrounding medium. A knowledge of the biology, including the ultra structure of fish spermatozoa is important for understanding and designing of preservation procedures for the gametes. Most of the fish species which have been subjects for sperm cryopreservation, are all external fertilizing teleosts with anacrosomal aquasperm. Cryoinjures during cryopreservation procedure often occur. The success of cryopreservation depends on minimizing the extent of cryoinjures during freezing and thawing.

CRYOPRESERVATION PROTOCOL

I. Milt collection

Milt is a suspension of spermatozoa in the seminal fluid. Sperms of fishes are immotile when they are within the testis and in undiluted form. Activity is only initiated by dilution with water/saline or ovarian fluid. Several factors may be responsible for the immotility but not clearly understood, possibly O2 depletion, a protein androgammone? pH, or KCl con. (more of K+ inhibits and Na+ activate). In general, spermatozoa of fresh water and saline water spawners are activated by hypotonicity and hypertonicity respectively of their surrounding media. Motility is considerably shorter in fresh water spawners because of osmotic injury. There is positive correlation between motility and fertility and motility is often used as an indicator of fertility in artificial insemination, because it is easy to assess microscopically. Sperms taken from properly stored dead fish are viable for 1½ to 5 hours.

Oozing male fish can be stripped manually by applying gentle pressure on the abdomen. All aseptic precaution should be taken and also care should be exercised to avoid contamination with blood, urine, scales and faeces. An intramuscular/intraperitoneal injection of 'Ovaprim' 3ml/kg body weight can be given 8 hours prior to stripping.

II. Evaluation of milt

Evaluation of milt quality is done by placing a drop of milt in a glass slide and its motility checked in sea water. A cover slip is carefully placed over it and observed the motility of sperms at the inter-junction of milt and sea water in a compound microscope at a magnification of 10x. Three main types of movement could be observed: (1) rapid, progressive or shooting movement, (2) sluggish or lethargic movement and (3) vibration *in loco*. A quick estimation of the approximate percentage of spermatozoa belonging to each category can be made and accordingly a motility score (1–5) can be given. Only samples with a motility score of 3 and above are fit for cryopreservation.

Criteria for motility score

Criteria	Score
90% or above of the sperms exhibiting rapid progressive or shooting movement	5
75% or more exhibiting rapid progressive, 10% sluggish and the rest immotile	4
50% exhibiting rapid progressive, 25% sluggish and 10% vibrating *in loco* and the rest immotile	3
25% exhibiting shooting movement, 50% moving sluggishly, 10% vibrating *in loco* and the rest immotile	2
occasional sperm shooting, 10% showing sluggish movement, 50% vibrating *in loco* and the rest immotile	1
completely immotile	0

III. Dilution

a. Extender

Milt has to be diluted before cyopreservation. A suitable extender containing a cryoprotectant may be used for this purpose. An extender is a solution of balanced salts and some times organic compounds mimicking the composition and osmolality of seminal plasma. One of the functions of the salt or organic compounds is to inhibit osmotically the activation of the sperm motility. As motility of sperm depends on internally stored ATP which can be resynthesised only at very slow rates, the extender must ideally inhibit sperm motility before freezing since fertility declines after activation probably due to sperm exhaustion.

Composition of some of the commonly used extenders

Extender Composition (mg)	CC1 (Kurokura *et al*, 1984)	Rana and Mc Andrew (1989)	Chao (1975)	Marine teleost ringer solution	Mixture B(Elizabeth, 1987)	Mixture C(Elizabeth, 1987)	V_2E (Scott& Baynes, 1980)
NaCl	750	650	1350	1350	600	600	750
KCl	20	300	60	60	38	38	38
$CaCl_2$	20	30	-	-	-	23	-
$NaHCO_3$	20	20	20	20	200	100	200
$NaHPO_4$	-	-	-	-	-	41	-
$MgSO_4$	-	-	35	35	23	23	-
$MgCl_2$	-	-	-	-	-	-	-
Na_2HPO_4	-	-	-	-	53	-	-
Glucose	-	-	5000	-	-	-	100
Egg yolk	-	-	-	-	-	-	20
Water	100	100	100	100	100	100	100
pH	7.3	7.3	7.2	6.8	7.0	7.3	7.0

b. Cryoprotectant

Unprotected cells can survive freezing if they are cooled at optimal rates, but generally cooling rate low enough to prevent intracellular freezing is low enough to produce lethal exposure to high concentrations of electrolytes. The major role of cryoprotectants is to bind electrolyte and thereby preventing these substances from concentrating in the residual unfrozen solution in and around cells during freezing. Additionally they bind with water molecules and reduce pure crystal formation. They also lower freezing point of intracellular fluid to –45°C. To be effective, a cryoprotectant must permeate into the cell, otherwise it would not prevent a rise in intracellular electrolytes during freezing. However, some macromolecules which cannot enter cells have been shown to protect against freezing damage.

Cryoprotectants can suppress most of the cryoinjuries, but when used at higher concentrations most of them become toxic to biological materials. Their toxicity should be less than their protective ability or they would damage rather than protect the cells. Choice of cryoprotectant is normally on the basis of toxicity and permeability to cells and solubility in water during freezing. Though the optimum concentration may vary between cryoprotectants, species and equilibration time used, a final concentration between 7 and 15% have been successfully used in most cases. Cryoprotectants can be divided into two groups – those permeable to cell membrane and those not permeable.

(i) Permeating cryoprotectants

Commonly used permeating cryoprotectants are dimethyl sulphoxide (DMSO), glycerol, methanol and 1,2 – propanediol. Of these compounds, the glycerol is the least toxic to most biological material but also the least permeable to cell membrane and hence takes longer to equate with glycerol osmolality. Methanol on the other hand is highly permeable to cell membranes, but it is generally considered most toxic. DMSO is fairly permeable to membranes but its toxicity intermediate between glycerol and methanol and can be minimized by reducing the temperature. For this reasons, DMSO is the most widely used permeating cryoprotectant.

Concentration of cyoprotectants used

Sl. No.	Cryoprotectants	Concentration (%)
1	DMSO	7.5, 10, 12.5
2	Glycerol	5, 10
3	Methanol	5, 10
4	Propylene glycol	8, 12
	Combinations:	
5	DMSO and Glycerol	5+5
6	Methanol and Glycerol	5+5
7	DMSO and Propylene glycol	5+5
8	Methanol and Propylene glycol	5+5

(ii) Non-permeating cryoprotectant

Non-permeating cryoprotectants include sugars (glucose, sucrose), polymers (polyvinyl pyrolidone) and proteins (egg yolk, serum proteins, skimmed milk). Their protective ability usually in conjunction with a permeating cryoprotectant, is related to their ability to depress freezing point and prevent ice crystal formation. Specific lipids with potential for lowering membrane phase transition temperature are used to minimize membrane damage during initial cooling and during freezing.

The milt is generally mixed with cryodiluent in the ratio of 1:3. All solutions are to be maintained at 20°C.

IV. Equilibration time

Equilibration time is the time after adding the diluents (extender+cryoprotectant) and before freezing. During this period the cryoprotectant penetrates the cells and equilibrates with the surrounding media. Fish spermatozoa may become motile upon mixing with diluents, hence the equilibration time is kept minimum to avoid sperm exhaustion. This precaution will in turn, minimize cryoprotectant toxicity. As spermatozoa are sufficiently small and DMSO penetration is rapid, no lengthy equilibration period is required for this cryoprotectant. However, penetration of glycerol is slow and a longer equilibration is required. By vacuum equilibration technique equilibration time can be shortened greatly by augmenting penetration of cryoprotectant, at the same time minimizing toxicity. With the exception of glycerol, permeability of most cryoprotectants is not markedly reduced by low temperatures and therefore, equilibration is usually performed at O°C to reduce cryoprotectant toxicity. Exposure to glycerol is preferably done at room temperature.

Equilibration time of 10 mts over ice is to be given including the time to fill the diluted milt into 0.5 ml French straws. Fill the straws and seal with polyvinyl alcohol powder.

V. Freezing and storage

Milt prepared from previous phase is cooled from 0 to −196°C during this phase. It should be noted that even with the use of protective agents, there is still an optimum cooling rate, though a very wide one for fish spermatozoa. For freezing of fish spermatozoa, a two step procedure is generally applied – milt is cooled in liquid nitrogen(Ln) vapour on a floating tray or hanging in the in the neck of the container and then straws are plunged into the Ln. The cooling rate is determined by the height of the tray or the depth at which the straws are lowered in Ln. The cooling rate successfully used is 30–160°C/minute.

Storage temperature is usually −196°C in Ln. Maintenance of this temperature is essential and spermatozoa can be stored indefinitely without appreciable deterioration.

VI. Thawing

At this phase gametes are warmed from storage temperature to above O°C. Rapid thawing is necessary to avoid recrystalization. Thawing rates in marine fish are 10–40°C/minute. Thawed spermatozoa must be used immediately. Generally the motility rate of freeze–thawed spermatozoa is high in marine fish species compared to fresh water species. In marine species, the high motility rate of frozen-thawed spermatozoa also results in high fertilization rate. The duration of motility in post–thawed sperm markedly reduces. Cryoinjuries have been reported in thawed spermatozoa of many fish species, but relatively less in marine fish. The better ability to withstand the rigors of cryopreservation may be due to the lipid composition of sperm membranes, mainly the molar ratio of cholesterol to phospholipids which is two or three times higher than the freshwater fish.

VII. Post–thaw evaluation

The motility of frozen thawed semen is a reliable parameter for quality determination, since there is statistically greater correlation with post–thaw fertilization rate. Post-thaw motility assessment can be carried out by rapidly plunging the straws into water bath at 37°C for 20 sec. The sealed ends of the straws are cut open to expel the thawed milt. A small drop of thawed milt taken on the glass slide and mix with seawater and immediately observe under the microscope(10x). Post-thaw motility can be judged by two variables – (a) percentage of motile spermatozoa on a 5 point scale (b) duration of motility using a count up timer.

After thawing, spermatozoa may show different motility characteristics from untreated sperm. The duration of motility can be markedly reduced from few minutes to few seconds. Only an empirical quantification of actual fertilization is a reliable indicator of success of cryopreservation procedure.

Artificial insemination & *in vitro* fertilization

Gametes that have survived the cryopreservation procedure are ready for artificial insemination. Artificial insemination has to be performed immediately after thawing, since the delay will reduce the fertility rate. The speed of sperm movement depends on temperature. Since the energy reserve of fish spermatozoa is limited, the increased speed caused by rise in temperature shortens the life span. The lowest limit for natural spawning (0-10°C) is generally used as the temperature for insemination.

An insemination medium has to be given for adequate distribution of spermatozoa in relation to ova. Freshwater or seawater are not the best medium for freshwater or marine fish sperm respectively as morphological changes takes place in these media. Insemination media best adapted to dilution of fresh and marine fish sperm have a salinity of 4-7% and 20% respectively and pH value about 9 in both types. In these solutions sperm injuries are minimal.

The best fertility of fish spermatozoa diluted in insemination medium can be obtained at an optimal sperm concentration and fertility declines more rapidly when the dilution exceeds 1:10 (v/v). Not all spermatozoa survive cryopreservation procedure and densities of frozen-thawed sperm have to be higher than for fresh sperm to maintain the same level of fertility.

Conclusion

Simple cryopreservation protocols are available for marine fish spermatozoa. The extender generally consists of saline or sugar solution in which DMSO added as cyoprotectant. Methodologies such as exposure of straws on tray in the vapour, immersion in Ln and thawing in water bath are used for freezing and thawing. To date, the semen of about 30 different marine fish species have been cryopreserved and a high survival of frozen-thawed spermatozoa is often recorded. Work on marine species have mostly been concentrated on the improvement in freezing technique protocols, but lacked studies involving morphological and metabolic changes. Also, the quality of sperm sample before freezing should be investigated in more detail, as the problem of semen aging and urine contamination can alter the biological features of the spermatozoa and their suitability for freezing. The possible improvement in sperm fitness for cyopreservation by modifying rearing parameters during spermatogenesis (water temperature/food composition) has not yet studied in marine fish species.

The cryopreservation technique for spermatozoa of marine fish is applicable for production purposes in aquaculture, as well as for establishment of gene banks. Coupled with insemination, cryopreservation will lead to an improvement of gamete management in Mariculture.

Suggested reading

1. Jamieson, B.G.M. (1991) Fish Evolution and Systematic Evidence from Spermatozoa. Cambridge University Press

2. Harvey, B.J. and S.J.de Croos (2000) Cryopreservation of gametes in Aquatic Species. Aquaculture Research (Special Issue), Volume 31, Number 3, March 2000.

CRYOPRESERVATION OF FISH GAMETES

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Introduction

Conservation and sustainable utilization of natural resources are issues receiving worldwide attention after signing the Convention on Biodiversity. Unbridled exploitation of resources has crossed the sustainable levels and has led to extinction of a number of species of plants and animals. Human interference has disturbed ecosystem of many water bodies. A few of then remain in pristine condition. In several parts of the world, there is growing awareness among the beneficiaries and user-agencies on the imperative need to conserve, protect and manage various ecosystems. Therefore, there is an urgent need for conservation of natural resources in our country also.

The genetic resource conservation as conceived and proposed by UNEP (1980) includes two essential requirements:

Conservation *in situ*: Conservation of genetic resources through their maintenance within natural or man-made ecosystems in which they occur.
Conservation *ex situ*: Conservation outside their habitats either by perpetuating sample population in genetic resources centre or in the form of gene pools of gamete storage (cryopreserved milt, eggs, embryo), germplasm banks etc.

Biomass conservation concept: In addition to *in situ* and *ex situ* conservation, the recent approach is the biomass conservation concept. It is entire preservation of vast tracts of habitats with *in situ* conservation, which will be extremely important in slowing the rate of species extinction.

Cryopreservation of fish gametes: Successful freezing of fish gametes and to store them for long periods without deterioration would be of considerable value in the improvement of aquaculture. It will have immense applied value and would open new perspectives in establishing genetic material reserves for selective breeding, provide a means of protecting endangered species by gene banking, for time-independent distribution of genetic material, to conduct cross breeding of desirable stocks and to improve existing induced breeding technology etc.

Prolonged storage of aquatic eggs and embryos has met with little success but it should not be impossible in view of recent scientific developments in this regard. Techniques have, however, been developed for spermatozoa preservation of many teleosts fishes which would undoubtedly need be modified for other species.

Principles of Cryopreservation

The basic principles of cryopreservation are discussed at different stages of a generalized cryopreservation procedure as follows.

A. Freezing:

When cells are cooled in an aqueous solution, both cells and solution supercool to some extent; then heterogeneous nucleation takes place, usually in the extracellular solution. If it should occur intracellularly, the resultant nuclei will be isolated by plasma membranes from the other unfrozen cells. As water is frozen out, the extracellular solution becomes progressively more concentrated. If the cooling rate is low, there is sufficient time for the cells to lose enough water to remain in osmotic equilibrium with the concentrating extracellular solution. However, prolonged exposure to concentrated solution is generally lethal.

If the cooling rate is high, there is insufficient time for water to diffuse out of the cells to the ice crystal. The cells will equilibrate by intracellular freezing initiated either by homogeneous or heterogeneous nucleation. Intracellular freezing is considered fatal. If the cell is sufficiently small, not exceeding a few microns, as in microorganisms, so much water can be withdrawn during freezing that they are effectively desiccated.

A balanced situation may exist which allows survival when the cooling rate is high enough to minimize the time of exposure to concentrated solution and yet is low enough to minimize the amount of intracellular ice below a damaging level. Certain chemicals can increase the dimension of this balance between the effects of intracellular ice and concentrated solution, thus improving survival. These chemicals termed cryoprotectants.

It is possible to predict such optimum cooling rates using mathematical models provided that values of factors required for the models are known. These factors are: (1) the viscosity of the intracellular solution; (2) the surface to volume ratio of the cell; (3) the rate of diffusion of water through the cell membrane; (4) the distance between the cell and the nearest ice crystal; (5) the viscosity of the extracellular solution; and (6) the permeability of the added cryoprotectant. An extremely high cooling rate theoretically allows survival if the thawing rate is equally high. Under these conditions, intracellular nuclei may form but have insufficient time to grow or glass transformation may take place with little nucleus formation. However, these extreme rates of cooling and warming are not feasible for biological material. High freezing and thawing rates (510-760°C/min) are necessary for survival of the membranes of rat liver mitochondria.

B. Thawing

During thawing, the same physicochemical processes take place in reverse order. Theoretically, the thawing rate should be the same as the corresponding cooling rate. However, there is usually a minute amount of small intracellular ice present even when an optimum cooling rate is used. Recrystallization invariably occurs during thawing, forming lethal intracellular ice. A high warming rate is usually employed to minimize the degree of recrystallization. When thawing is rapid, there is insufficient time for the dehydrated cells to absorb the amount of water lost during freezing. Most cells and tissues appear to be tolerant to rapid thawing, mammalian embryos constituting a notable exception. Rapidly changing solute gradients cause membrane damage to mouse embryos during rapid thawing.

C. Storage

Storage temperature should be -130° C or below and a commonly used storage temperature is -196° C (the temperature of, liquid nitrogen). This is simply because liquid nitrogen is a convenient storage medium. At these low temperatures, all biological molecules become motionless and, therefore, cannot participate in any biochemical reactions. Theoretically biological material could be kept indefinitely in this frozen state.

However, reactions at atomic level can still take place at the temperature of liquid nitrogen. Of these reactions, decomposition of cell nuclei caused by background radiation is the major concern in cryopreservation because it is potentially mutagenic. DNA damage caused by background radiation is cumulative since no DNA repair would take place during storage at -196° C. However, mouse embryos exposed to the equivalent of about 2,000 years of background radiation did not show observable deterioration, excepting those with' mutant genes or chromosome anomalies. Under most circumstances, background radiation is of no importance in cryopreservation and the period of storage under these conditions is almost indefinite.

D. Cryoprotection

The addition of some chemicals (cryoprotectants) can minimize cell damage associated with ice formation or, when used at high concentrations, will suppress any ice formation. Such a process is termed cryoprotection.

D.1. Cryoprotectants

Some commonly used cryoprotectants are listed below:

Chemical	Molecular Formula	Molecular Weight	Density
Dimethylsulphoxide	$CH_3SO\ CH_3$	78.13	1.10
Glycerol	$CH_2OH\ CHOH\ CH_2OH$	92.10	1.47
Methanol	CHOH	32.04	0.79
1,2-Propanediol	$CH_3CHOH\ CH_2OH$	76.09	1.04

Water solubility and low toxicity are the most important properties of the cryoprotectants. Cryoprotectants must be highly water-soluble in order to alter the physicochemical properties of water during freezing. Their toxicity, if any, must be less than their protective ability, or they would offer damage rather than protection. Cryoprotectants can be divided into two groups: those permeable to the cell membrane, and those not permeable.

D.1.1. Permeating Cryoprotectants

Permeating cryoprotectants serve (1) to reduce the rate of diffusion of water from the cell to the ice crystal; (2) to reduce the amount of cell volume change/salt concentration colligatively; (3) to lower the homogeneous nucleation temperature; (4) to re- duce the rate of ice crystal growth; and (5) to raise the glass transformation temperature. Choice of the cryoprotectant is normally on the basis of toxicity and permeability to the cells, and solubility in water during freezing.

Commonly used permeating cryoprotectants are DMSO, glycerol, methanol and 1,2-propanediol (Table above). Of these compounds, glycerol is the least toxic to most biological materials but also the least permeable to the cell membrane and hence takes longer to equilibrate with glycerol osmolality. Nevertheless it was found to give equal or superior protection to the deleterious effects of freezing and thawing on mouse embryos when compared with DMSO. The large difference between the permeabilities of water and glycerol causes volume effects during introduction and removal of this cryoprotectant. Methanol, on the other hand, is highly permeable to cell membranes but is generally considered the most toxic, excepting a few cases of remarkably low toxicity DMSO is fairly permeable to membranes but its toxicity, intermediate between glycerol and methanol, can be minimized by reducing the temperature; it is also, like glycerol, a radioprotective agent. Experiments using radiolabeled cryoprotectants show the degree of permeation of methanol, dimethyl sulphoxide [DMSO] and glycerol is inversely proportional to the molecular weight of the compounds; glycerol does not penetrate the egg, while methanol, which penetrates with the greatest rapidity, achieves no more than 23% of the expected equilibrium concentration after 2 h exposure at 0°C.

Unlike glycerol, the permeability of DMSO, is not markedly affected by low temperature. Probably for these reasons, DMSO is the most widely used permeating cryoprotectant. A relatively recent addition to the list of cryoprotectants, 1,2-propanediol (propylene glycol), is possibly better than DMSO. Its success is due, at least partly, to the fact that it has a higher glass formation tendency than glycerol or DMSO as indicated by scanning differentiation calorimetry studies.

D.1.2. Nonpermeating Cryoprotectants

Non-permeating cryoprotectants include sugars (e.g. sucrose, glucose), polymers (e.g. dextran, hydroxyethyl starch, polyvinylpyrrolidone, PVP) and proteins, (e.g. egg yolk, serum, skim milk, and the antifreeze proteins found in polar fish and in freeze-resistant and freeze-tolerant insects). These compounds, since they do not penetrate into cells, should not be able to promote any colligative protection. Their cryoprotective ability, usually in conjunction with a permeating cryoprotectant, is related to their ability to depress the freezing point and to raise the glass transformation temperature of the extracellular solution. Specific lipids, with potential for lowering membrane phase transition temperatures, are used to minimize membrane damage during initial cooling (i.e. cold shock) and during freezing.

D.2. Cryoprotectant Toxicity

Cryoprotectants can suppress most cryoinjuries but, when used at higher (more effective) concentrations, most of them become toxic to biological materials. Dimethylsuphoxide (DMSO) inhibits catalase and peroxide activity; ethylene glycol decreases the polarity of the aqueous phase and changes the partition of hydrophobic molecules between the cell membrane and the external phase; the resultant dehydration of the phospholipid bilayer causes membrane damage; DMSO and polyethylene glycol, in combination, are exceedingly dangerous, inducing membrane fusion; toxicity of methanol causes failure of hearts to recover after thawing if frozen to below 30°C. Toxicity from high concentrations of DMSO, however, can be suppressed by addition of amides.

Cryopreservation of milt

Milt should be stripped into dry tubes without contamination by urine or faeces. Fish sperms come out live, but they are inactive. This is because of the high potassium ion concentration in the milt plasma. The moment they come in contact with water, or a solution having high pH (above 8.0), they all become active and the motility lasts only for few seconds to few minutes depending on the species followed by osmotic shock and death. There is no use of preserving sperms that are already active, as during the freeze-thaw protocol their activity will be lost. Hence it is necessary to dilute the milt with an "extender solution" before freezing. The extender solution is a mixture of salts at an appropriate pH (usually 7.0 to 7.5), which helps to maintain the viability of the spermatozoa during refrigeration without activating them. The extender solution containing a cryoprotectant (combination is usually called "cryo-diluent"), is thought to bind electrolytes during the freezing process, thus preventing them forming lethal concentrations. They also lower the freezing point of the intracellular fluids.

Glycerol, ethylene glycol and propylene glycol have been used as cryoprotectants for fish sperm, but the most widely effective is dimethyl sulphoxide (DMSO). The rates of freezing and thawing, however, need be established independently for each species. Freezing rates of 30-160°C per minute have generally been successful. Thawing is generally accomplished by dropping the frozen milt directly onto the eggs.

Method for marine fish milt cryopreservation

The method developed for marine fish milt cryopreservation can be adopted with modifications for other species.

i) Freshly stripped milt is mixed in the proportion 1:3 with extender cryoprotectant solution held at same temperature as the milt.

ii) 200 ml aliquots of extended milt are frozen on a block of solid CO_2. A 0.5 cm diameter depression is drilled into the top surface of the CO_2 block. Using a repeater pipette 200 µL of milt mixture is poured in it. Frozen pallets are stored in freezer vials in liquid nitrogen.

iii) An alternative method using only liquid nitrogen is to fill mini straws of fine plastic tubing with 250µL or 500 µL aliquots of extended milt. The ends are plugged and frozen above liquid nitrogen. An approximate cooling rate is achieved by placing the straws of milt on a thin metal tray supported 4 cm above the surface of the liquid nitrogen by a polystyrene float. For this purpose liquid nitrogen can be placed in a polystyrene igloo box. The straw, after 10 minutes, is immersed in the nitrogen and then stored until required.

iv) The frozen milt in liquid nitrogen can be stored for a long time since the rate of deterioration is negligible.

v) Best fertilization rates are obtained in rapid thawing of the frozen milt. 5 pellets of milt are added to 5 ml of seawater at ambient temperature, shaken for a few seconds until they are slushy and then added immediately to the eggs for fertilization. Alternately 2 straws are thawed in a water bath at 40°C for 5 seconds, the ends cut off and the

contents added to eggs simultaneously with 5 ml of seawater. These quantities of milt are sufficient to fertilise 500 to 1000 eggs.

Extender solution : The extender solution for the marine fish milt is a mixture of :

Distilled water	100 ml
Sucrose	4.28 g
KHCO3	1.00 g
Reduced glutathione	0.20 g

7 parts of this solution is added to 1 part of dimethyl sulphoxide

The factors, which may require modification for freshwater and other species, include extender composition, extent of semen dilution, freezing and thawing rates, number of spermatozoa required per egg and dilution of milt during fertilization.

MOLECULAR BIOLOGICAL BASIS OF IMMUNE RESPONSES IN FISHES

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Introduction

The term immunity means exemption. This meaning was derived from exemption granted to certain categories of citizens in the case of compulsory military services. It has been recognized that those who recovered from epidemic diseases were exempt (immune) from further attacks. This knowledge helped Edward Jenner in 1798 to develop vaccine against small pox. Immune responses have its evolutionary beginning in animal kingdom some 400 million years ago. These responses have maintained a remarkable constancy at molecular and functional levels. The basic pattern of the protein molecules involved in this process has been maintained (conserved), while at the same time diversification of molecules to suit the emerging challenges was super imposed on the basic pattern. The immune phenomenon is believed to have evolved from the basic mechanism of self-recognition and non-self discriminations. Self-recognition and non-self discrimination appear very early in evolution and can be seen in marine organisms such as sponges. Aggregation of dispersed colonies of sponges is regulated by species-specific surface glycoproteins. Failure of adhesion of unrelated species amounts to a primitive form of graft rejection. The cells lining the cavity of sponges are able to capture microorganisms. Phagocytosis plays a role in the metamorphosis of insects in removing dead and disintegrated tissue.

Pollination and subsequent fertilization involves again recognition molecules. The same mechanism is involved in the fertilization of ova of marine fishes and other organisms by the spermatozoa of same species. Sea stars and corals are able to reject graft of unrelated forms. The self-non-self discrimination enables the organism to maintain specific association between its own millions of cells, while excluding changed self-constituents and potentially harmful parasitic organisms, such as protozoa, fungi and bacteria.

Basic pattern of Molecules

The cell surface molecules that are markers of self/non-self recognition are either carbohydrate or carbohydrate terminal groups on glycoproteins. The recognition factors themselves are proteins. Many of the marker proteins and recognition proteins have evolved either from β2 micro globulin or Thy-1 protein. The basic structures of immunoglobulin, histocompatibility antigen and phagocyte receptors indicate this. Each β2 micro globulin consist of polypeptide chain with110 amino acids of MW 12000 (Approximate). The vertebrates including fish have improved upon this primitive recognition mechanism and evolved a highly efficient system to deal with potential invasion from the co-existing biological world as well as aberrant or rogue cells evolved through spontaneous mutations (egs. Cancer). This system has retained all the primitive mechanisms such as, Phagocytosis,

agglutination of heterologus cells, lysis etc., while developing specific molecules and specific cellular mechanism against foreign molecules and cells. Hence, the immune system of vertebrates has two branches *viz.* innate immune system and acquired immune system; where as in invertebrates only the innate immune system exists.

The innate and acquired immunities are two branches of immune system, which are intimately interlinked. It is not possible to compartmentalize them into two separate units. Many times the help of one is required for the functioning of the other or one stimulates and modulate the other system. For example antibodies enhance the Phagocytosis of innate system. Antigen-antibody complex activates complement system. Interferons and interleukins stimulate and modulate acquired immune responses.

Innate immunity: non-specific defense mechanisms

The innate immune mechanisms are non-specific, since they are effective against a wide range of potentially infective agents. The main determinants of innate immunity are genetically controlled, varying widely with species, strain and to a lesser extent between individuals.

Surface barriers: 1. Mucus 2. Skin/ Exoskeleton. 3. Gills. 4. Gastro-intestinal tract.
Mucus: A layer of mucus (glycoproteins, proteoglycans and proteins) forms the interface between body and environment. Mucus entraps microorganism and mucus is continually replenished by mucus secreting cells, which inhibits the colonization of integument. The rate of secretion of mucus increases in response to infection or due to action of irritants. Lysozyme, bacteriolysin and complement cascade present in fish mucus are anti-microbial.

Skin: The skin surface of fish differs from that of higher vertebrates in that the epidermis composed of non-keratinized living cells. Epidermal integrity is vital to fish in maintaining osmotic balance and extending microorganisms. The epidermal healing response in fish is extraordinarily rapid, even at low temperatures. It involves a migration of malpighian cells from the periphery of wound surface rapidly closing the lesion, and is quite different from the scab formation, which occurs in mammals. Epidermis has resident migratory phagocytes. Malpighian cells are also capable of migration and Phagocytosis.

Exoskeleton: Crustaceans have exoskeleton made of chitin, which is frequently replaced.

Gills: Comprising such a large surface area of delicate epithelium, the gill is considered to be an important route of entry of microorganisms. The organ is protected by mucus production and a highly responsive epithelium resulting in hyperplasia, frequently seen in many infections. Pillar cells, that line brachial blood sinus are phagocytic.

Gastrointestinal tract: The lining of the tract is a mucous membrane, which secretes mucus in copious amounts. The digestive function of the gut provides an extremely hostile environment to pathogens. (1) Acidic pH in stomach (2) Action of digestive enzymes

[trypsin and pepsin]. In teleosts fish M cells and Peyer's patches are absent. However intraepithelial lymphocytes and macrophages together with eosinophilic granular cells situated in lamina propria are present.

Non-specific humoral factors: These include (1) growth inhibitors (2) inhibitors of enzymes or toxins produced by the pathogen (3) lysins (4) precipitins and (5) agglutins.
Growth inhibitors: These substances act either by depriving microorganism of essential nutrients or by interfering with their metabolism.

Metal ion binding proteins: These occur in the serum of all vertebrates including fish. Iron binding proteins (siderophilins) such as apotransferrins, ceruloplasmin and metallothionein inhibit the growth of bacteria. All these have been identified in fish. Apotransferrin binds two ferric ions. Ceruloplasmin oxidizes ferrous ions to ferric ions and metallothionein binds metal ions such as copper, zinc, cadmium and mercury. Metallothionein specifically binds to macrophage plasma membrane, initiating respiratory burst activity and signal transduction. Apotransferrins, which are also acute phase proteins, display anti -microbial properties by limiting the amount of endogenous iron available to pathogens including intracellular bacteria/ protozoan.

Acute phase proteins: Plasma proteins collectively termed as acute phase proteins increase in response to infections, and tissue injury. These include C-reactive proteins, serum amyloid A protein α_1 antitrypsin, α_2 macroglobulin, fibrinogen, ceruloplasmin, C_9 & factor B.

Cytokines: Cytokine related molecules are detected in fish and invertebrates. These are interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6 tumor necrosis factor (TNF), chemotactic protein-1 macrophage migration inhibition factor (MIF) and other peptide factors, which are involved in modulation of immune response and inflammatory reactions. Cytokines mobilize the host immune response, activate inflammatory reactions, and mediate bi-directional communication among various organ systems. Colony- stimulating factors (glycoproteins and peptides) regulate haematopoiesis and haematopoietic cell function, and transforming growth factors profoundly affect wound healing and cellular differentiation.

Interferons: are proteins that inhibit intracellular viral replication. The interferon has been reported from fresh water and marine fishes. They are classified into class I interferon (include α and β interferons) and class II interferons (γ interferons). With in each type there are several different forms. α interferons are produced mainly by lymphocytes and other nucleated cells. Interferon β is produced by fibroblasts and interferon γ is produced by T-lymphocytes and natural killer cells (NK or NC cells). The interferon producing cells when infected with virus (stimuli for INF synthesis are nucleic acids, bacterial cell walls, double stranded RNA and poly-synthetic nucleotides) synthesize and secrete interferons into extra cellular fluid. The interferons bind to specific receptors of uninfected cells. The antiviral effect is produced by derepression of two genes leading to the synthesis of two specific enzymes. One-enzyme catalyses the phosphorylation of ribosomal protein and initiation

factor elf-2, necessary for protein synthesis. This reduces the m-RNA translation in cells. The other enzyme catalyses the formation of short polymer of adenylytic acid, which activates a latent endonuclease, this in turn, degrades viral and host cell m-RNA. This establishes a cordon of uninfected cells around the site of viral infection restraining its spread. In addition to these effects it has several other immunological function such as major histocompatibility class II protein molecule (MHC II) expression on macrophages, increased Phagocytosis by neutrophils and macrophages. It also enhances activity of natural killer cells, T-lymphocytes, B-lymphocytes and other immune cells.

Eicosanoids: These are an important group of compounds derived from 20 carbon polyunsaturated fatty acids. In fish, eicoanoids are generally produced in organs rich in blood cells and in blood cells after ionophore stimulation. These eicosaoids include prostaglandins, thromboxans, lipoxins and leukotrines. They regulate blood clotting, MHC II expression, inflammation and Phagocytosis.

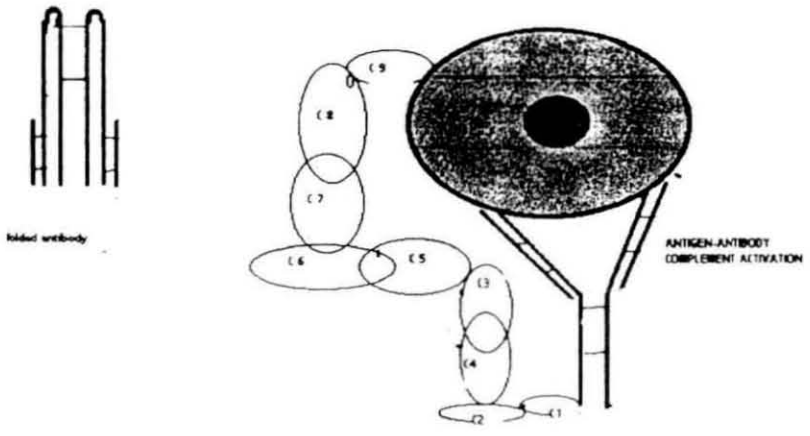
Enzyme inhibitors: Many pathogens produce enzymes in order to gain access to host body. Host tissue fluid and blood contain many factors, which neutralize these lytic enzymes. α_2-macro globulin inhibits a wide range of proteinases. α_2-macro globulin is able to entrap and form covalent linkages with proteins such as transforming growth factor (TGF) B_1, IL-1B and platelet derived growth factor BB. α_2-macro globulin thus regulates the action of coagulation cascades and complement cascade.

Lysins:

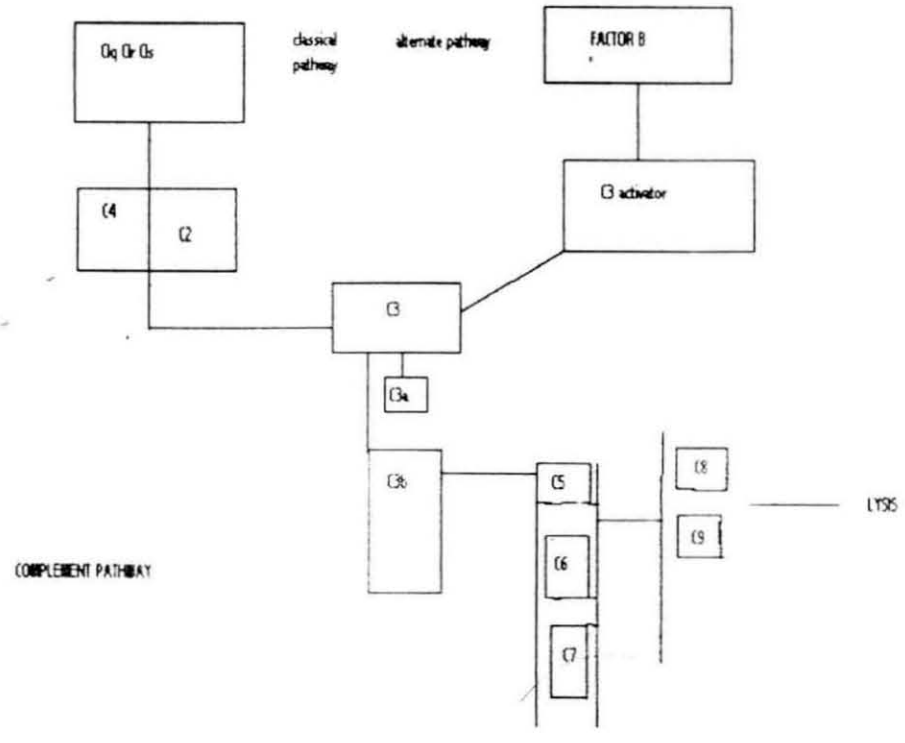
There are several enzyme systems, which cause lysis of heterogenous cells. These include complement cascade, pro-phenol oxidase, lysozyme and trypsin.

Complement: is an enzyme cascade system present in serum and tissue fluids, composed of twelve protein components. This is an extremely complex group of proteins. It has a broad spectrum of biological activity. Complement activation may be linked to humoral mediators of inflammation including the kinin and clotting systems

On the biochemical level many similarities exist between complement of fish and mammalian complement. Fish complement is highly heat labile and neutralized at 45°C. The complement can be activated by two major mechanisms. 1) The classical pathway in which the antigen-antibody complex exposes complement receptors on antibodies to which complement factors are adhered and enzyme cascade is triggered leading to formation of holes on target cells (antigen) and final lysis



2) Alternate pathway-the bacterial endotoxins, polysaccharides like zymosan and inulin, which activate properdin or factor B, which convert complement factor C_3 into C_{3b} and C_{3a} and thus the entire cascade is triggered into action.



In fish, complement is found in serum and mucus.

Pro-Phenol oxidase system: This system comprises an enzyme cascade leading to the activation of pro phenol oxidase and other compounds with related activities. Pro-phenol oxidase on activation by β-1, 3 glucans and the zymosan cleaves to phenol oxidase, which

catalyses oxidation of tyrosine to quinones. Quinones polymerize to form melanin deposits. Melanin deposition is a common immune response seen in invertebrates. Conversion of pro-phenol oxidase to phenol oxidase is done by a protease named phenol oxidase activating enzyme. This enzyme is kept in check by a protease inhibitor.

Lysozyme: This is a low molecular weight basic protein found in blood leukocytes of vertebrates, haemocytes of invertebrates, serum, haemolymph and mucus. It functions as a mucolytic enzyme, splitting sugars off the glycopeptides of the cell wall of many gram-positive bacteria, resulting in their lysis. Lysozyme also plays a role in the intracellular destruction of some gram-positive bacteria.

Trypsin: Trypsin and trypsin containing cells are found in epidermis, gills and intestine indicating local production and its secretion into mucus.

Precipitins and agglutinins

C-reactive proteins (CRP) and serum amyloid protein A (SA): These are plasma proteins and forms part of acute phase proteins. CRP binds to phosphoryl ester groups of bacterial cell wall, which contain phosphoryl choline. This binding is Ca^{++} dependent and activates complement. It has a structural analogy to limulin of horseshoe crab. CRP enhances the migration, Phagocytosis and respiratory burst of phagocytes. CRP can act as opsonin. CRP causes precipitation of heterologus proteins/ carbohydrates in non-immunized sera.

Serum amyloid protein A has got lectin binding property, by which they bind to bacterial cell wall glycoproteins

Agglutinins: These are a group of phylogenically conserved substances that are present in fish serum, mucus, bile and haemolymph of invertebrates. This group of substances includes lectins and other receptor specific substances. They act as opsonins and cause aggregation by binding to protein/ glycoproteins and/ or carbohydrate moieties that are free in solution or are constituent of microbes. Lectins are usually constitutive proteins or glycoproteins, which posses binding activity towards carbohydrate residues.

Cellular factors

Phagocytosis and the inflammatory response: various circulating and tissue fixed phagocytes rapidly engulfs any foreign particles, which enter animal body. In vertebrates they are the polymorphonuclear leukocytes and the macrophages; where as the haemocytes constitute the phagocytes of crustaceans and mollusks. The phagocytes contain digestive enzymes that degrade the ingested material. The phagocytes recognize, bind and ingest particulate materials. Recognition and binding take place through interaction of cell surface glycoproteins and cell wall carbohydrates of microorganism. More recently evolved mechanisms that utilize receptors in the phagocyte cell membrane for a part of the antibody molecule (Fc portion of antibody) and for a component of the complement (C3b).

Microorganism coated with antibody and complement thus adheres to the phagocyte and can then be ingested. Antibodies that enhance Phagocytosis are the opsonins.

Inflammation:

Inflammation is the dynamic process occurring in a viable tissue. It is the reaction of tissues to irritants/ disease causing agents. Inflammation begins following sub lethal injury to tissues and ends with the repair or healing of injured/ damaged tissue. . Following an injury the first sequence of change occur in local vascular system. These vascular changes are the result of release of pharmacodynamic amines from injured mast cells. The eosinophilic granular cells found in fish are believed to be the analogues to mast cells found in higher vertebrates. These cells are abundant in connective tissue of blood vessels as well as the stromal connective tissue, which form the structural framework of many organs and tissues. The bacterial products, physical and chemical trauma, products of damaged cells and complement factors released by immune response can produce injury to mast cells, which release the amines like histamine and serotonin. These amines induce increased blood flow and dilatation of capillaries. This increased blood flow through the area of injury result in redness (*rubor*). Dilatation of capillaries causes stretching of capillary fenestration, which allows colloids of the plasma to escape into the interstitial space. This results in increase in colloidal osmotic pressure, which attracts fluid contents of blood to tissue spaces, this may lead to swelling of the area (*tumor*) and the fibrinogen escaping with plasma proteins will be initiated to form fibrin mesh work. Dilatation of arterioles and pre capillary sphincters cause more capillaries to be opened. An increase in capillary and venule blood pressure is associated with dilation of vessels. Increased permeability of capillaries and venules leads to retardation of the flow and drop in blood pressure. The endothelial cells are activated to produce a lectin on their surface----selectin. The retardation of blood flow allows the heavy elements of blood to be distributed evenly. The leukocytes have on their surface the selectin receptor, which cause them to adhere to the endothelial layer. Then inside out signaling causes certain integrins on the leukocytes (β_1 and β_2 subunits) to gain affinity for molecules of immunoglobulin family; particularly those called **ICAM** (Inter Cellular Adhesion Molecules) on endothelial cells. These attachments help the leukocytes to stop, squeeze between endothelial cells and cross the blood vessel wall into the damaged or infected tissue.

Exudation of plasma/ serum

The changes in blood flow and the dilatation of capillaries and venules following enlargement of afferent arterioles lead to retardation of blood flow. This causes increased permeability and osmotic pressure change. Injury to cells leads to breakdown of macromolecules and they enter intercellular fluid resulting increased osmotic pressure. Loss of colloids into interstitial space through increased vascular permeability leads to fall in osmotic pressure of blood. Hydrostatic pressure at venules is increased due to vasodilatation of arterioles. Hence, there is increased accumulation of fluid at the tissue side; where as the

re-absorption of fluid from tissue is retarded due to fall in vascular osmotic pressure. The exudates formed will have plasma proteins including fibrinogen.

The exudates have the following functions. (1) Dilute the irritants. (2) Globulins are brought in contact with the irritants, which may neutralise the irritants (antibodies). (3) Fibrinogen in exudates forms fibrin scaffolding around the irritants, which will contain the spread of infection. The fibrin mesh will act as anchor for leukocytes to perform their functions.

Migration of leukocytes

Leukocytes emigrate to the tissues by amoeboid movement. The chemo taxis initiates this. Lipo-polysaccharides of bacterial cell wall released at the site are major chemo tactic agent. The cleavage products of complement, such as $\overline{C_{3a}}$, C_{5a}, C_{567}, lymphokines produced from stimulated lymphocytes and the products of granulocytes and monocytes all act as chemo tactic agents. Fatty acid derivatives derived from injured cell membranes such as leukotrines, 5 hydroxyeiosateraenoic acid (5HETE) are all chemo tactic.

White cells actively migrate through the fenestrae to enter the affected tissues. The cells penetrate junctions between endothelial cells and between basement membrane. They escape to the tissues at the points where basement membrane splits to accommodate pericyte. The collagenaese enzyme of leukocytes digests collagen. Lymphocytes are pinocytosed by endothelial cells and the vacuoles are exocytosed at the basement membrane region. Erythrocytes also leave through the fenstrae. The cells, which leave the blood, are 1) neutrophils, 2) monocytes, 3) eosinophils, 4) lymphocytes and 5) thrombocytes.

Neutrophils:

These are the first cells to migrate. They contain numerous cytoplasmic granules, which are lysosomes containing a number of enzymes capable of destroying the ingested organisms. They are hydrolytic enzymes, oxidative enzymes, proteolytic enzymes, phagocytin and lysozyme.

The proteolytic enzymes are two categories; acid proteases and neutral proteases. Acid proteases act with in phagosomes; where as neutral proteases degrade collagen, basement membrane, fibrin, elastin and cartilage. The neutral proteases are responsible for tissue destruction and this may release kinin and split complement factors C_3 and C_5 that in turn induces chemo taxis.

Opsonic serum factors coating on particles enable phagocytosis. They are complement fragments in fish. Immunoglobulin coated opsonisation is weak in fish. The phgocytosis by neutrophils release some quantity of enzymes to the tissues because the fish neutrophils are not efficient phagocytes. The phgocytosis and subsequent digestion are energy dependent. There are two types of digestion, oxygen dependent and oxygen

independent system. In oxygen dependent system there are two types. The superoxide system is characterized by increase in hexose monophosphate shunt activity (This is called respiratory burst, which can be demonstrated by Nitroblue tetrazolium staining-NBT). This generates superoxide anions O_3^-, H_2O_2, $^{\cdot}OH$ and $O^{\cdot-}$ These radicals affect macromolecules of the living organisms and thus kill organisms like bacteria. In the myeloperoxidase-peroxidase system myeloperoxidase enzyme increases the efficiency of H_2O_2 generating system by releasing halide ions (free halide). This system is more efficient in killing. In oxygen independent system---Hydrogen ions (H^+) reduce pH. Hydrolytic enzymes hydrolyses macromolecules; lysozyme split off sugars of bacterial cell walls. Fish neutrophils have very similar morphological and histochemical properties to mammalian neutrophils. They are present in kidney, spleen, blood and inflammatory lesions.

Monocytes

These cells appear in an inflammation in later stages. They are actively mobile and send numerous pseudopodia. The monocyte nucleus is ovoid, kidney shaped or indented. Nucleus is usually eccentrically placed. Nucleoplasm is condensed near its membrane. Cytoplasms are abundant and contain mitochondria, Golgi apparatus, rough and smooth endoplasmic reticulum. Once these cells reach tissues after leaving the blood stream they divide and mature. They are known as mononuclear macrophages. Their main function is phagocytosis. They can engulf large particles. They can recognise complement coated cells and particles through specific receptors that assist in phagocytosis. They also recognise molecules that have altered or denatured membranes and engulf them. They can secrete hydrolytic enzymes. Some of the macrophages mature into secretary cells with abundant cytoplasm and become closer to each other with indistinct boundaries. They are called the epithelioid cells. Differentiation to epithelioid cells occurs in chronic inflammation; where macrophages try to destroy irritants by secreting enzymes.

Mcrophages are wide spread in tissues but their concentration is more in reticulo-endothelial system. Reticulo-endothelial cells are found in interstitial tissue of kidney, spleen and endocardial lining of heart. Many macrophages in fish contain melanosomes within lysosomes. These are termed melano-macrophages. Melanin plays a role in bactericidal mechanism involving release of free radicals. Melano-macrophages form aggregates in parenchymal organs, which are called melanomacrophage centres.

Natural killer cells (Natural cytotoxic cells in fish)

Natural killer cells are large granular lymphocyte. They recognize structures on high molecular weight glycoproteins, which appear on the surface of infected cells. This recognition occurs through receptors on NK (NC) cells surface, which bring killer and target into close opposition. Activation of NK cells ensues and leads to release of granular contents into the space between two cells. The important factor perforin or cytolysin insert into membrane of the target cell and produce an annular pore. This induces cell death. The granules contain two serine estrases.

Eosinophils

Eosinophils are evolved to kill large parasites. A major basic protein MBP is located in the core of the granules. Cationic protein and peroxidase are present in the matrix of granules. Other enzymes are arylsulphatase B, phospholipase D and histaminase. It also contains dopaminase. Eosinophils have receptors for C3b. Activation produces respiratory burst and generation of oxygen metabolites. One of the granules can produce a trans-membrane plug like perforin. Most helminthes activate alternate pathway. The C3b allows eosinophils to adhere and activated eosinophils secrete MBP and cationic protein.

Haemocytes of in vertebrates have the same enzymes described for leukocytes and perform the phagocytic and degradation function seen in vertebrate cells.

Acquired immunity

Acquired immunity has two wings. (1) humoral immunity (2) cellular immunity or cell mediated immunity. Fish has developed both these systems.

Humoral immunity. The characteristic of this form of immunity is the appearance of globulins-immunoglobulins or antibodies in blood. These antibodies combine specifically with the antigen, which stimulate their production and lead to remarkable consequence.

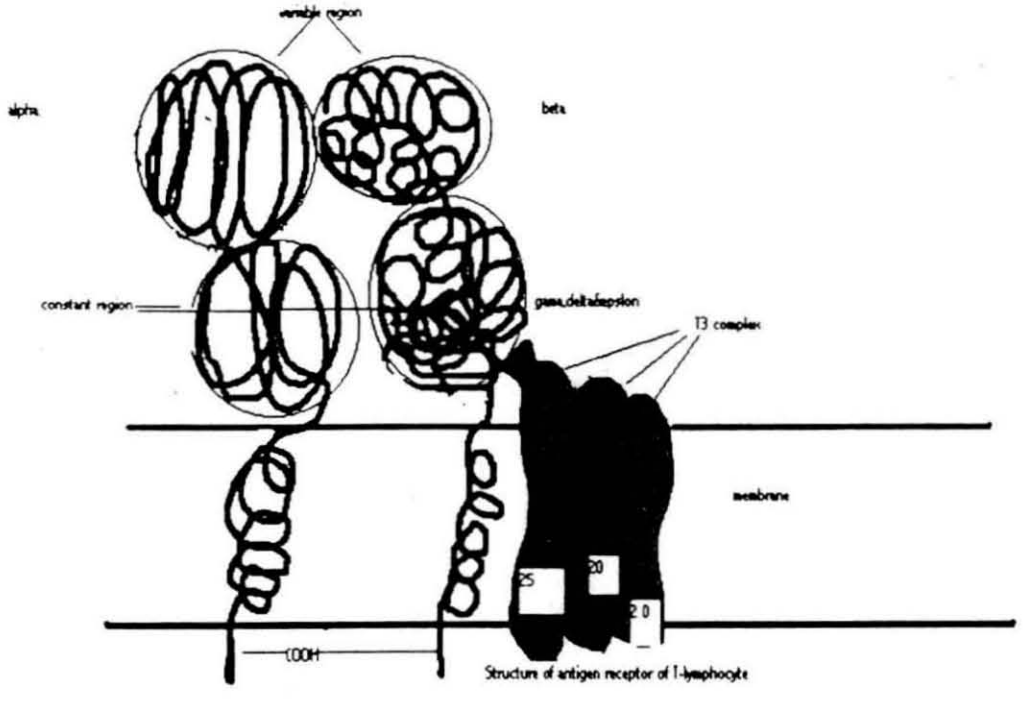
Cell mediated immunity (CMI).

Lymphoid cells may be induced, by prior exposure to antigen, to react subsequently directly with the inducing antigen and bring about cytotoxic effects, as for example on foreign cells from a graft. In both type of immunity the executors of the reaction are lymphocytes. Lymphocytes are fond in the circulation, the lymphoid organs and other tissues. In mammals and birds there are two distinct type of lymphocytes (1) originating or primed in thymus—T lymphocytes (2) bone marrow or bursa of Fabricius derived cells—B-lymphocytes. T lymphocytes are responsible for cell mediated immune responses (CMI), providing assistance for antibody production (helper function) and suppression of immune reaction.

B-lymphocytes are executors of humoral immunity. They transform into antibody or immunoglobulin secreting cells on antigenic stimulation. Subsets of T&B cells perform another function i.e. memory of any antigen encounter. In fishes dichotomy of immune system is also present but the details are not fully worked out. Evidence for T&B lymphocytes is available.

Lymphocyte subpopulations. The T lymphocytes have surface antigen receptors, which are α and β receptors in adult and δ γ receptors in embryonic stage. T lymphocyte receptor is a hetrodimer composed of α and β chain each of molecular weight 40-50 kD. Each chain

is folded into two domains, one having a relatively in constant structure, the other exhibiting far more variability. The variable region has the job of binding to antigen and MHC.



Both α and β chains are required for antigen specificity. In all immuno-competent T lymphocytes, the antigen receptor is non-covalently but still intimately linked in a complex with T_3, a molecule composed of three peptide chains (γ δ ε), which transduces the antigen recognition signal received by $\alpha\beta$ heterodimer to the inside of cell. The complete receptor is thus consisting of five peptides. In fish, thymocytes have a portion of Ig of heavy chain. In mammals no heavy chain receptor is found in thymocytes, where as they bear light chain related molecules.

T lymphocytes are differentiated in thymus. In higher vertebrates the priming of lymphocytes to T lymphocytes occurs. Thymus is protected against the ingress of foreign antigens by specialized endothelial lining of the blood vessels. The role of the thymus in fish is probably similar to that of mammals. The thymus consists of mainly lymphocytes in various stages of development and a few epithelial cells and macrophages. There is no differentiation into cortex and medulla. Thymus is a paired organ occupying dorsal region of brachial cavity and is extremely superficial, being situated with in the epithelium external to basement membrane. The blood vessels of thymus have specialized endothelium with tight junctions. In the embryo this is the first organ become lymphoid. A single layer of epithelium covers the thymus up to post fingerling stage. There are fenestration of 20mμ diameter in the epithelial layer. In older fish these fenestrations close and epithelium become thickened or thymus become deeply embedded in underlying tissue. Involution of thymus starts at sexual maturity, but it is a slow process in fish. Even in older fish some amount of thymic tissue will be discernable. Thymus is a primary non-executive lymphoid organ. Foreign particulate matter and protein antigen present in circulation are not able to

enter thymus lymphocytes also do not enter thymus. There is high mitotic activity in thymus and there is migration of thymocytes from thymus to spleen, anterior kidney and intestine. In adult fish thymus is responsible for regulating antibody response to T dependent antigen and suppressor activity. The developing thymocytes exhibit membrane antigen, which decide their future roles. These antigens are extensively studied in mouse. The mouse T cell antigens are termed Thy (τ) TL and Ly antigens. TL antigen is lost and the amount of Thy is reduced during differentiation. Ly antigen appear later in the development. Ly-1, 2,3 antigen expressing cells are the immature T-cells. Ly1, 2, 3-cell cell give rise to Ly-1 cells, Ly-2, 3 cells and Ly-1Qa$^+$ cells (60% Ly-1 cells are bearing Qa$^+$). Ly-1 cells are having helper function and Ly-1Qa$^+$ cells control the generation of suppressor cells. Ly-2, 3 cells have suppressor function on helper cells and B-cells. Ly-2, 3 cells also function as T-killer cells or cytotoxic cells. In other vertebrates the helper function is associated with CD4 receptor bearing cells and killer and suppressor function is associated with CD8 receptor. CD4, 8 cells are immature T-cells. Though clear-cut division of based on T cell antigen has not studied in fish thymus cells, the helper, killer, and suppressor functions are noticed in fish lymphocytes. Antigen difference between thymus lymphocytes and lymphocytes of anterior kidney are also evident. In mammals T lymphocyte antigen cross-reacts with brain tissue antigen. In fishes the lymphocytes, which are responsive to T-cell mitogens have antigens cross-reacting with brain tissue antigens.

B-cells have antigen receptors as single heavy chain of μ (Ig M). The B cell is produced in lymphoid tissues like anterior kidney and spleen. The B lymphocyte on binding with respective antigen through the receptor is stimulated to undergo multiplication and many of these cells acquire immunoglobulin secretary function.

Response to mitogens: Certain plant lectins are found to specifically stimulate division in lymphocytes. Phyto-haemagglutinin (PHA) and Concavalin-A (Con.A) are T-cell mitogen, where as lipopolysaccharides specifically stimulate B-lymphocytes. In fishes it is found lymphocytes cross-reacting with brain tissue antigen are responsive to PHA and Con.A, where as they are not responsive to lipopolysaccharides. Lipoplysaccharide responsive cells bear Ig M heavy chain molecule on their surface; where as PHA-Con.A responsive cells bear only a part of heavy chain Ig molecule.

The carrier hapten effect: One method to detect T-helper function is to estimate carrier hapten effect. Certain low molecular simple substances when injected into an animal will not produce an antibody response. If this low molecular substances is chemically linked to a large molecule and injected it will induce antibody response against it. The molecule, which is attached to the large molecule, is the hapten and the molecule, which is carrying it, is the carrier. Dinitro-phenyl (DNP) molecule will not induce immune response, where as; if it is linked to bovine serum albumin, it will elicit antibody response against DNP. In carrier hapten effect T-cell co-operation with B cell is required. Carrier hapten effect has been demonstrated in a number of fishes. It is also noticed that T lymphocytes of fish are capable forming rosettes with sheep erythrocytes.

Cell mediated immune reactions in fishes:

The markers of CMI are allograft versus host reaction. In graft versus host reaction an organism rejects organ/ tissue transplant from individual of the same species as well as from phylogenically different species. The rejection process will be faster, if the donor and recipient are genetically non-related. In this type of reaction no antibody is involved but only lymphocytes and macrophages. Once the animal reject a tissue transplant it will reject another transplant from the same donor at a short duration of time, thus rejection reaction induces immunological memory in recipient.

Scale transplantation and skin transplantation (both allograft and xenogrft) have been attempted in fish. In all cases rejection and immunological memory have been noticed. The lymphocytes of the recipients have been shown to retain sensitivity to donor antigens.
Delayed hypersensitivity reactions: These are lymphocyte-mediated reactions and lymphokine mediated reactions. These are specifically provoked by slowly evolving mixed cellular reactions involving lymphocyte and macrophages. The reaction is not brought about by circulating antibody but by sensitized lymphocytes. And can be transferred in experimental animals by means of such cells not by serum. The classical example is the tuberculin response. The animals or humans infected with *Mycobacterium tuberculosis*; 0.1 ml of 1 in 1000 dilution of protein extract of *Mycobacterium tuberculi* is given intradermally. An indurated inflammatory reaction in the skin appears about 24 hrs later and persists for weeks. The injection site is infiltrated with large number of lymphocytes and macrophages. Most of these cells are seen around small blood vessels. Among circulating lymphocytes there are a few sensitized lymphocytes, which on contact with antigen produce lymphokines and influence other lymphocytes and monocytes to aggregate at the site of antigen concentrations; and lymphocyte multiply at this site.

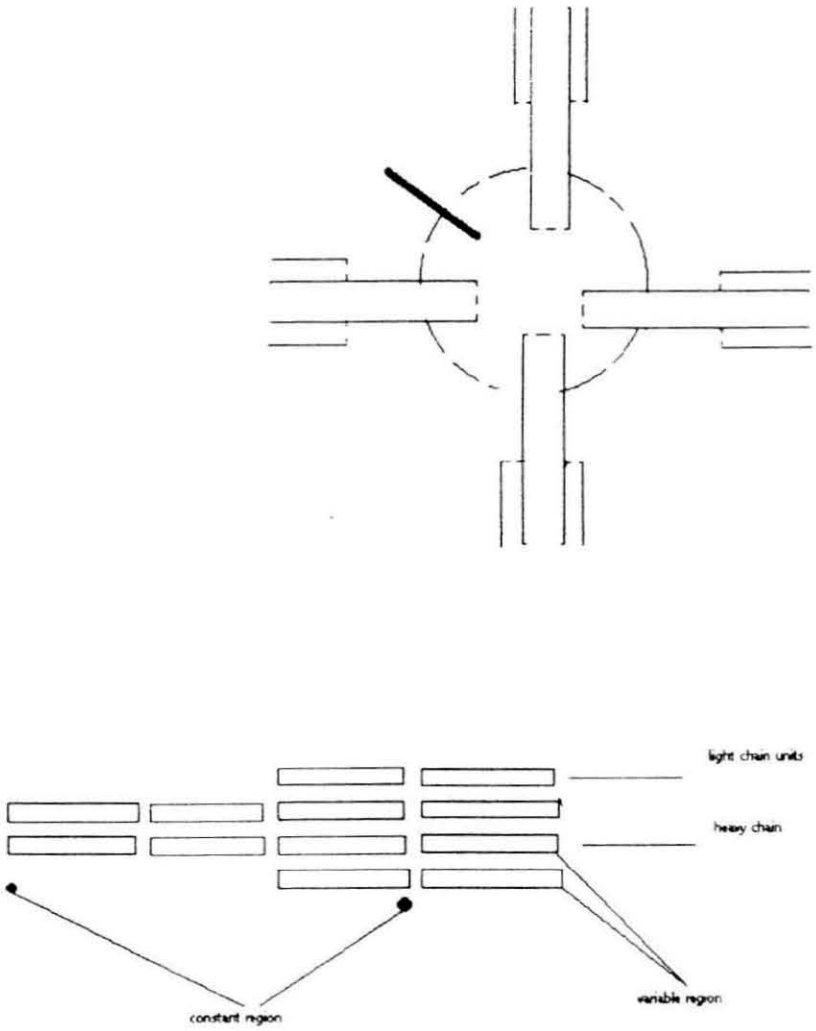
Delayed hypersensitivity can be demonstrated in fish by injecting T dependent antigens like BSA or tuberculin and making lymphocytes sensitized. Later an intradermal injection of the antigen will produce local inflammatory reaction like one described above. In chronic disease like bacterial kidney disease of trout delayed hypersensitivity has been demonstrated.

Thus it is clear fish has got a very good CMI response and the cells analogues to T lymphocytes of mammals are present in fish. The production of lymphokines in fishes can also be demonstrated by test like macrophage migration inhibition test and demonstration of chemotaxis in special chambers. Macrophage activation by the lymphokines can also be demonstrated. *In vitro* tests like specific contact cytotoxicity, mixed leukocyte reaction and antigen-induced blastogenesis of lymphocytes indicate fish has a strong CMI.

Humoral immunity

Presence of antibodies can be demonstrated in fish sera by agglutination, precipitation and complement fixation tests. In mammals and birds there are five classes (isotypes) based on the antigenic difference in the heavy chains. The basic structure of immunoglobulin consists of two heavy chains and two light chains. The heavy chain classes are μ,α,γ ε and δ (IgM IgA, IgG, IgE and IgD). The light chains are two types λ (lambda) κ (kappa). The basic structure is shown below. In mammals serum IgM is a pentamer, which consists five basic units linked in the form of a ring attached with protein called J segment. In teleost only IgM isotype is found. The serum IgM is tetrameric. Monomeric and dimeric form of IgM is also seen in mucus, bile skin and eggs.

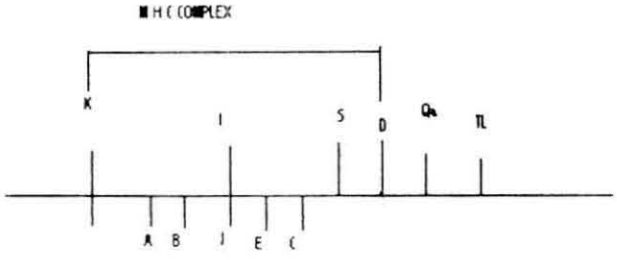
In mammals functional specialization is associated with heavy chain classes. In teleost antibodies can execute most of the reactions observed in mammals indicating heterogeneity at the level of function exist? It was also noticed with in species heavy chain molecular masses exhibited heterogeneity. There is also increasing evidence of local synthesis of secretary antibodies in bile, cutaneous and gastrointestinal mucus. Though immunological methods demonstrated only one type of antibodies in fish, there is structural difference with in the heavy chains. The differences may not have been amplified sufficient to produce antigenic differences in immunological reactions to classify them into different idiotypes.



BASIC STRUCTURE OF IMMUNOGLOBULIN-POSSIBLE EVOLUTION IN VERTEBRATES. THE MOLECULE IS 12 UNIT STRUCTURE. EACH CONSISTS OF OF POLYPEPTIDE CHAIN WITH 110 AMINO ACIDS OF MW 12000. EACH LIGHT CHAIN OF CONSISTS OFTWO BASIC UNIT AND HEAVY CHAIN HAS FOUR BASIC UNITS

Cell co-operation and major histocompatibility complex. The precise mechanism by which the immuno-competent cells co-operate involves the cell surface antigens. These antigens are glycoproteins and those involved in the rejection of grafts and these on transfer of a graft of tissue to unrelated recipients are recognized as foreign. The cell surface antigens are known as histocmpatibility antigen as the **major histocompatibility complex (MHC).** The genes controlling MHC are closely related to immune response genes and they are situated very close to immune response gene (Ir gene) loci in the same chromosome. The MHC genes have been studied in many mammals and rainbow trout. As a

model we take mouse. In mouse these genes are situated in chromosome number 17 and the graphic representation is given below.



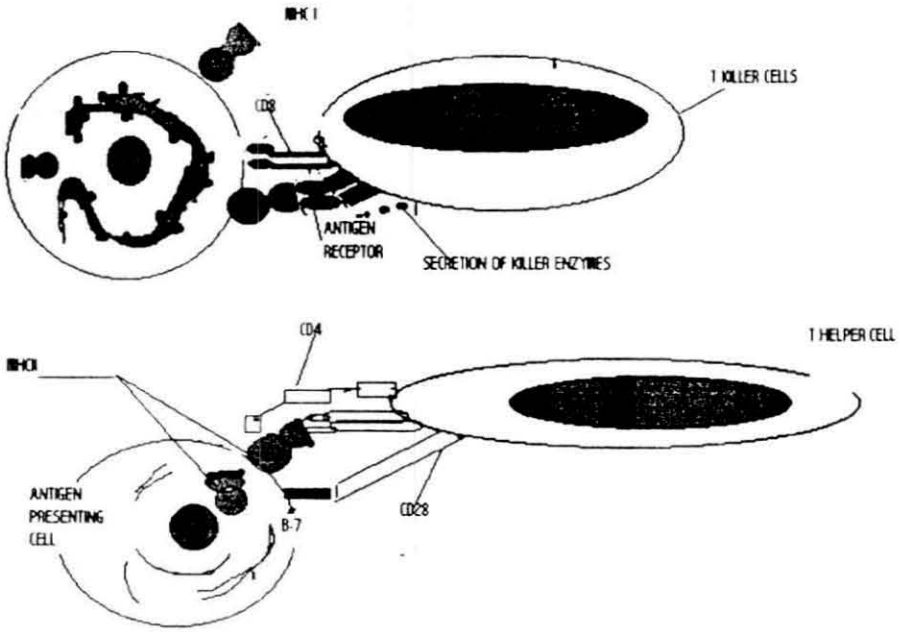
MHC COMPLEX	H2					Th	
MHC CLASS	1	2	3	1		1	1
GENETIC REGION	K	I	S	D		Qa	Th
GENES	K	ABJE	C4 SLP C2 Factor8	D	L	Qa-17	Th
GENE PRODUCTS	H2K	I-A, I-E, I-J	C4 C2 FB	H20 H-24		Qa 2-5	Th Qa1,6

The histocompatibility genes/ antigens belong to two classes. They are major histocompatability complex II and I. The genes coding for C4 and factor B, have made their way into the MHC region and are referred to as class III genes. Both class I and class II molecules are membrane bound heterodimers. Class I molecule consists of a heavy chain of 43 KD non-covalently linked to a smaller 11 KD peptide-β_2 micro globulin. The heavy chain has the globular domains α_1 α_2 and α_3, which protrude from cell surface. The hydrophobic section anchors the molecule into cell membrane and short hydrophilic end, which is C terminus, enters the cytoplasm.

The class II MHC is also trans-membrane glycoproteins having α and β polypeptide chains with molecular weight of 34 KD and 28 KD respectively. Both chains are folded to give two domains the ones nearest to membrane having considerable homology with β_2 micro-globulin and the characteristic Ig domain. It is seen that the I-J region code for more number of peptides than the space for genes it can hold, which that loci can hold. This includes suppressor and helper T lymphocyte receptors, immuno- globulins and other peptides. Probably the gene alleles mediate the selection of structurally related molecules indirectly, perhaps through idiopathic interactions involving T cell receptors. In the immunoglobulin system variability is achieved in each individual by a multigenic system. In MHC variability is achieved between individuals with highly polymorphic system based on

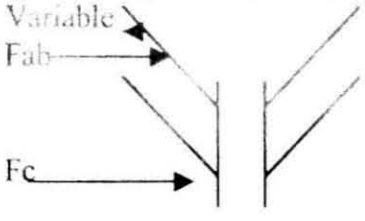
multiple alleles. It is very high in class I molecules. Multiple allelic forms can be generated by variety of mechanisms, point mutation, recombination, and homologous but unequal crossing over and gene conversion. This mechanism has been detected in mice. Most of the mutations contain clusters of multiple amino acid substitution and seem to arise by transfer of up to 95 nucleotides from class I Qa genes to$\alpha_1$and α_2 domains of H-2K. These findings have indicated that the large number of functionless Qa genes may represent a stockpile of genetic information for the generation of polymorphic diversity in the working class I molecules. Evidence for gene conversion has also been obtained for the class II genes.

All nucleated cells express class I molecules. These are abundant on lymphoid cells, less so on liver kidney and only sparsely on brain and striated muscles. Class II molecules are restricted to B-lymphocytes, macrophages and antigen presenting cells; however when activated by cytokines capillary endothelium and many epithelial cells express class II. MHC molecules have several physiological functions in addition to immunological functions. Here, we are more concerned immunological functions. It is known fact that detection non-self MHC activates the T-lymphocytes. The T-cell can recognize an antigen in association with MHC. The class I molecule is synthesized in the rough endoplasmic reticulum and transported to cell surface in a transport vesicle The MHC molecules have a groove which carries a native peptide (β_2). The T cells with CD8 receptors search the MHC I molecules. In viral infected cells the native peptide is replaced by viral encoded peptides or in cancer cells and old cells the peptide has different constitution. The detection of changed configuration in MHC leads to the multiplication and secretion of cytotoxic molecules by CD8 cells. Hence destruction of the cell occurs. The MHC class II also has a groove. The molecule synthesized in RER but held in cytosol. In B-cell the antigen captured by surface antibody receptor is delivered inside the cell. The antigen is broken down into small peptides; the class II molecule grabs the peptide and moves to surface (macrophage also degrades the antigens and fragments are exhibited on MHCII). The CD4 T- lymphocytes combine with the antigen presented on MHC II molecule. In an infection the B cells and macrophages express another molecule B-7. This combines with CD 28 on the T-cells. These bindings trigger T cells to secrete cytokines which initiate B-cell proliferation and antibody secretions. In the absence of non-production of B-7 molecules the T-cells become inactive or anergized.



Clonal Selection Theory: This theory proposes that the cells of the antibody-forming system have developed from random mutations resulting in the emergence of small numbers of cells or clones of cells differentiated so as to be capable of producing one or a very small number of specific antibodies. Contact by such differentiated cells with self or foreign antigens during fetal life before cells have reached maturity, would lead to suppression because the cells are annihilated by apoptosis.

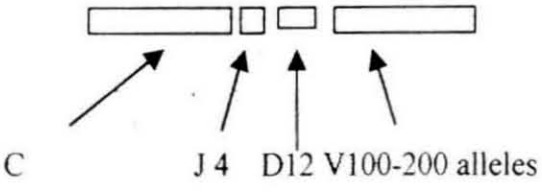
After initial contact with antigen the cells of immune system retain memory, subsequent contact with antigen lead to quicker as well as amplified immune response. The immune system becomes more skilled with continued experience with the same antigen. The antibodies increase its combining capacity-avidity. This is due to the expansion of the clone. Memory involves the generation of long-lived T and B cells and changes in receptor involving generation of high affinity receptors bearing cells.



B-lymphocytes generate so many different antigen receptors. The antibody genes, particularly the variable region polypeptide genes are inherited as gene fragments. These fragments are joined together to form a complete gene in individual lymphocytes as they develop. The joining process it self generate more diversity. The enzymes that combine gene segments add random DNA bases to the ends of the process being joined as a result new genes are formed. Further diversity results from the assembly of protein chains into

complete receptor. Antibodies are made from two pairs of protein chains a heavy chain and light chain. The heavy chains are connected to form a **Y**, with the light chain located on the upper branches, along side the heavy chain. Each B-cell produces just one kind of light chain and one kind of heavy chain so that each B-cell makes unique receptor. The Genes for receptors of B-cells mutate extremely rapidly, when antigens activate B-cells.

Each heavy chain and light chain has variable (V) and constant (C) domain. The V domain is in the N terminal of both chains. V domain chain gene in heavy chain is formed by the recombination of four fragments-J, D and V. J for joining with C and D the diversity segment. The V region forms hyper variable region. V has 100 alleles in mouse, D 12 and J 4. These are randomly assembled in lymphocytes with non-coding introns in between. The introns spliced in m RNA during this splicing operation further diversity introduced by addition and deletion of nucleotides. The same mechanism is used in the case of light chains and generation of T-cell receptors.



PROGRAMMED CELL DEATH—APOPTOSIS IN HEALTH AND DISEASE

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Introduction

The health of multicellular organism depends not only on the body's ability to produce new cells but also on the ability of individual cells to destruct when they become superfluous or disordered. Millions of cells are sacrificed every hour. This critical process is essential for the development and maintenance of multicellular organism; is termed apoptosis. The significance of apoptosis was overlooked for decades. But biologists have recently made rapid strides in understanding how cellular suicide is enacted and controlled. It is known that aberrant regulation of apoptosis-leading to too much or too little apoptosis-probably contributes to disorders like cancer, AIDS, Alzheimer's disease and autoimmune diseases (rheumatoid arthritis, lupus erythromatosis).

Death is not bad for the body always. In fact it is necessary for the health and well being of multicellular organism. The tadpole deletes its tail during transformation to frog; the arthropods delete several appendages/tissues during metamorphosis; mammals erase countless neurons as the nervous system takes shape. Human embryo deletes the web between digits during development. The lens of eye, which forms during embryonic development, consists of apoptotic cells that have replaced their internal contents with protein crystalline. Cells composing intestinal villi arise at the base of the villi and over several days, travel to the tip. They die there and are sloughed off. Skin cells begin life in the deepest layers and then migrate to the surface, undergoing apoptosis along the way. The dead cells forms skin's protective layer. The cells lining uterine wall perish by apoptosis during menstruation. The cells that become infected by virus or sustain irreparable genetic mutation often kill themselves. T lymphocytes during the developmental stage encountering an antigen die by apoptosis.

Necrotic death occurs when a cell is severely injured, by physical injury or by oxygen/ nutrient deprivation. This death has the following features. The cell and its internal organelles undergo severe swelling/ ballooning due to failure of ion pumps. Another hallmark of necrosis is inflammation bringing white blood cells to the site of necrosis. Inflammation also damages the normal tissue in the vicinity. Apoptosis, or programmed cell death, is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells

play an active role in their own death (which is why apoptosis is often referred to as cell suicide). Cells that are induced to commit suicide: shrink, have their mitochondria break down with the release of cytochrome c, develop bubble-like blebs on their surface and have the chromatin (DNA and protein) in their nucleus degraded. The chromatin breaks into small, membrane-wrapped, fragments. The phospholipid phosphatidylserine, which is normally hidden within the plasma membrane, is exposed on the surface. This is bound by receptors on phagocytic cells like macrophagesand dedritic cells, which then engulf the cell fragments.

The [illegible] specific signals [illegible] biochemical and morphological changes occur in the cell. A family of proteins known as caspases is typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular substrates that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus. The result of these biochemical changes is appearance of morphological changes in the cell.
Some of these changes are illustrated in Figure 1, which shows time-lapse microscopy images of a trophoblast cell undergoing apoptosis.

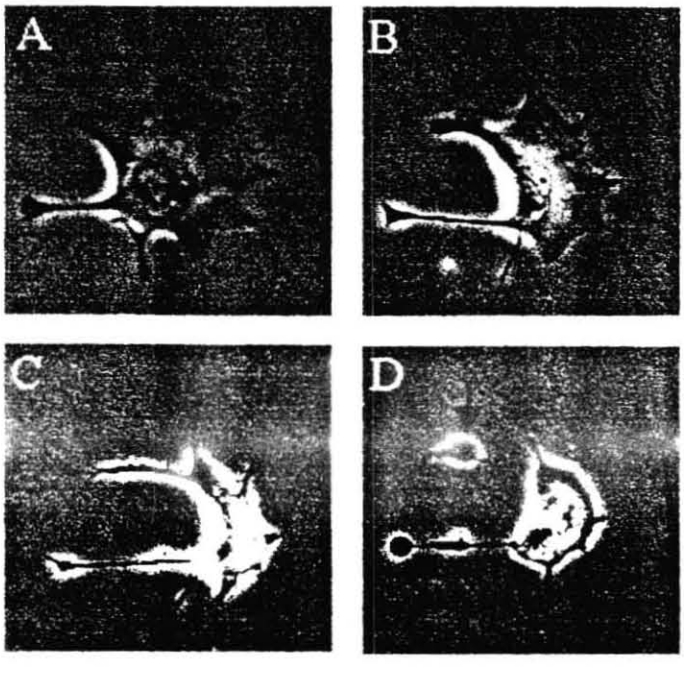

Fig1

Typically, the cytoplasm begins to shrink following the cleavage of lamins and actin filaments (A). Nuclear condensation can also be observed following the breakdown of chromatin and nuclear structural proteins, and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance (B). Cells continue to shrink (C), packaging themselves into a form that allows for easy clearance by macrophages. These phagocytic cells are responsible for removing apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often ungergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inner leaflet of the cell to the outer surface. Membrane changes can often be observed morphologically through the appearance of membrane blebs (D) or blisters, which often appear towards the end of the apoptotic process. Small vesicles called apoptotic bodies are also sometimes observed (D, arrow). In apoptosis cell spends energy, as it is an active process initiated by cell's own cleaving enzymes, which are interleukin-1 converting enzyme like proteases (ICE like proteases).

The Mechanisms of Apoptosis

There are three pathways through which apoptosis mechanism is activated. One generated by signals arising within the cell. Another triggered by **death activators** binding to receptors at the cell surface. These are TNF-□ Lymphotoxin and Fas ligand (FasL). A third that may be triggered by dangerous reactive oxygen species

Apoptosis triggered by internal signals: the intrinsic or mitochondrial pathway

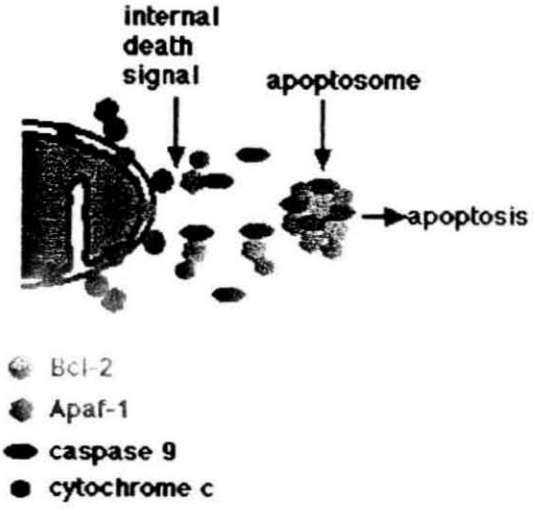

Fig.2

In a healthy cell, the outer membranes of its mitochondria express the protein **Bcl-2** on their surface. Bcl-2 is bound to a molecule of the protein **Apaf-1**. Internal **damage** to the cell (e.g., from reactive oxygen species) causes Bcl-2 to release Apaf-1 to no longer keep cytochrome c from leaking out of the mitochondria. The released cytochrome c and

Apaf-1 bind to molecules of **caspase 9**. The resulting complex of **cytochrome c, Apaf-1, caspase 9** (and ATP) is called the **apoptosome**. These aggregate in the cytosol. Caspase 9 is one of a family of over a dozen caspases. They are all proteases. They get their name because they cleave proteins - mostly each other - at aspartic acid (Asp) residues). Caspase 9 cleaves and, in so doing, activates other caspases. The sequential activation of one caspase by another creates an expanding cascade of proteolytic activity (rather like that in blood clotting and complement activation), which leads to digestion of structural proteins in the cytoplasm degradation of chromosomal DNA and phagocytosis of the cell.

Apoptosis triggered by external signals

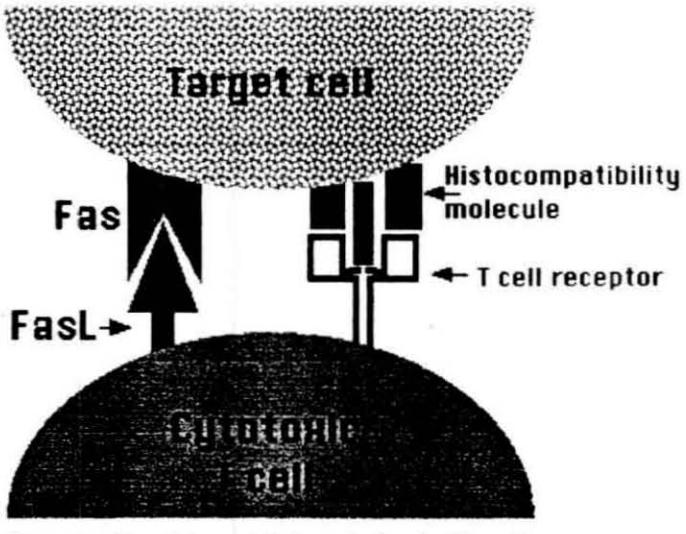

Fig.3

One method by which cytotoxic T cells induce their targets (e.g., virus-infected cells) to commit suicide (apoptosis)

Fas and the **TNF receptor** are integral membrane proteins with their receptor domains exposed at the surface of the cell. Binding of the complementary **death activator** (**FasL** and **TNF** respectively) transmits a signal to the cytoplasm that leads to activation of **caspase 8**. Caspase 8 (like caspase 9) initiates a cascade of caspase activation leading to Phagocytosis of the cell. When cytotoxic T cells recognize (bind to) their target, they produce more **FasL** at their surface. This binds with the **Fas** on the surface of the target cell leading to its death by apoptosis.

Apoptosis-Inducing Factor (AIF)

Neurons, and perhaps other cells, have another way to self-destruct that - unlike the two paths described above - does not use caspases. Apoptosis-inducing factor (**AIF**) is a protein that is normally located in the inter membrane space of mitochondria. When the cell receives a signal telling it that it is time to die, AIF is released from the mitochondria (like the release of cytochrome c in the first pathway) migrates into the nucleus and binds to DNA, which triggers the destruction of the DNA and cell death.

There are a number of mechanisms through which apoptosis can be induced in cells. The sensitivity of cells to any of these stimuli can vary depending on a number of factors such as the expression of pro- and anti-apoptotic proteins (eg. the Bcl-2 proteins or the Inhibitor of Apoptosis Proteins), the severity of the stimulus and the stage of the cell cycle. When cells are subjected to radiation injury or agents that induce mutation, the damage spurs the cells to a protein called p53. This protein activates the suicide pathway.

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In some cases the apoptotic stimuli comprise extrinsic signals such as the binding of death inducing ligands to cell surface receptors **(1)** or the induction of apoptosis by cytotoxic T-lymphocytes by granzyme **(4)**. The latter occurs when T-cells recognise damaged or virus infected cells and initiate apoptosis in order to prevent damaged cells from becoming neoplastic (cancerous) or virus-infected cells from spreading the infection.

In other cases apoptosis is initiated following intrinsic signals that are produced following cellular stress. Cellular stress may occur from exposure to radiation **(2)** or chemicals or to viral infection (3). It might also be a consequence of growth factor deprivation or oxidative stress. In general intrinsic signals initiate apoptosis via the involvement of the mitochondria **(5)**. The relative ratios of the various bcl-2 proteins can often determine how much cellular stress is necessary to induce apoptosis.

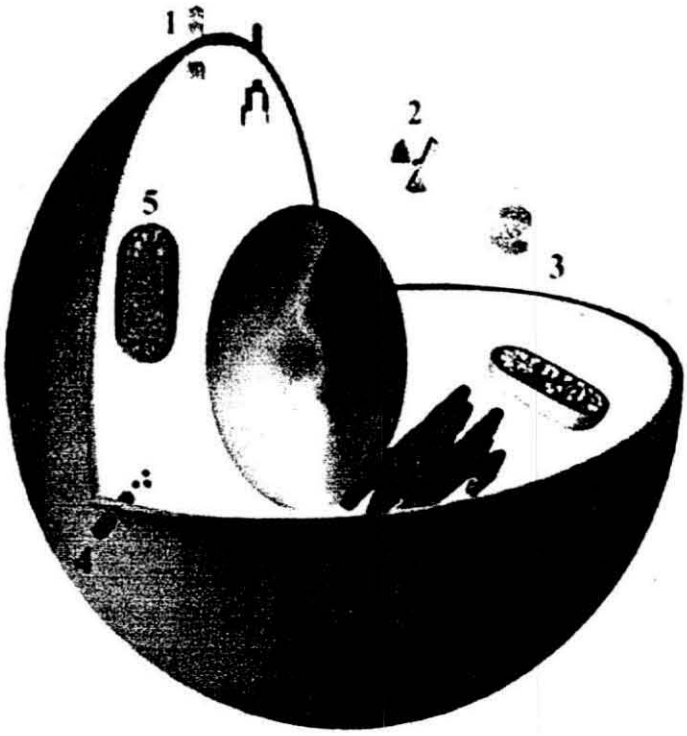

Fig.4

Death receptors

Death receptors are cell surface receptors that transmit apoptosis signals initiated by specific ligands. They play an important role in apoptosis and can activate a caspase cascade within seconds of ligand binding. Induction of apoptosis via this mechanism is therefore very rapid. Death receptors belong to the tumour necrosis factor (TNF) gene superfamily and generally can have several functions other than initiating apoptosis. The best characterised of the death receptors are CD95 (or Fas), TNFR 1(TNF receptor-1) and the TRAIL (TNF-related apoptosis inducing ligand) receptors DR4 and DR5. Signaling by tumour necrosis factor receptor 1 (TNFR1).

TNF is produced by T-cells and activated macrophages in response to infection. By ligating TNFR1, TNF can have several effects (seeFigure). In some cells it leads to activation of NF-kB and AP-1, which leads to the induction of a number of proinflammatory and immunomodulatory genes. In some cells, however, TNF can also induce apoptosis, although receptor ligation is rarely enough on its own to initiate apoptosis as is the case with CD95 ligand binding.

Viral diseases and cancer

Two human papilloma viruses (**HPV**) have been implicated in causing cervical cancer. One of them produces a protein (E6) that binds and inactivates the apoptosis promoter **p53**. **Epstein-Barr Virus** (**EBV**), the cause of mononucleosis and a cause of Burkitt's lymphoma It produces a protein similar to Bcl-2 and produces another protein that causes the cell to increase its own production of Bcl-2. Both these actions make the cell more resistant to apoptosis (thus enabling the cancer cell to continue to proliferate). Even cancer cells produced without the participation of viruses may have tricks to avoid apoptosis.

Some **B-cell leukemias** and lymphomas express high levels of **Bcl-2**, thus blocking apoptotic signals they may receive. The high levels result from a translocation of the *BCL-2* gene into an enhancer region for antibody production **Melanoma** (the most dangerous type of skin cancer) cells avoid apoptosis by inhibiting the expression of the gene encoding **Apaf-1**.

Some cancer cells, especially lung and colon cancer cells, secrete elevated levels of a soluble "decoy" molecule that binds to FasL, plugging it up so it cannot bind Fas. Thus, cytotoxic T cells (CTL) cannot kill the cancer cells.

Other cancer cells express high levels of **FasL**, and can kill any cytotoxic T cells (CTL) that try to kill them because CTL also express Fas (but are protected from their own FasL).

Apoptosis and Organ Transplants

For many years it has been known that certain parts of the body like, the anterior chamber of the eye and the testes are "immunologically privileged sites". Antigens within these sites fail to elicit an immune response. It turns out that cells in these sites differ from the other cells of the body in that they express high levels of **FasL** at all times. Thus antigen-reactive T cells, which express **Fas**, would be killed when they enter these sites.

This finding raises the possibility of a new way of preventing graft rejection. If at least some of the cells in a transplanted kidney, liver, heart, etc. could be made to express high levels of FasL, they might protect the graft from attack by the T cells of the host's cell-mediated immune system. If so, then the present need for treatment with immunosuppressive drugs for the rest of the transplant recipient's life would be reduced or eliminated. So far, the results in animal experiments have been mixed. Allografts engineered to express FasL have shown increased survival for kidneys but not for hearts or islets of Langerhans.

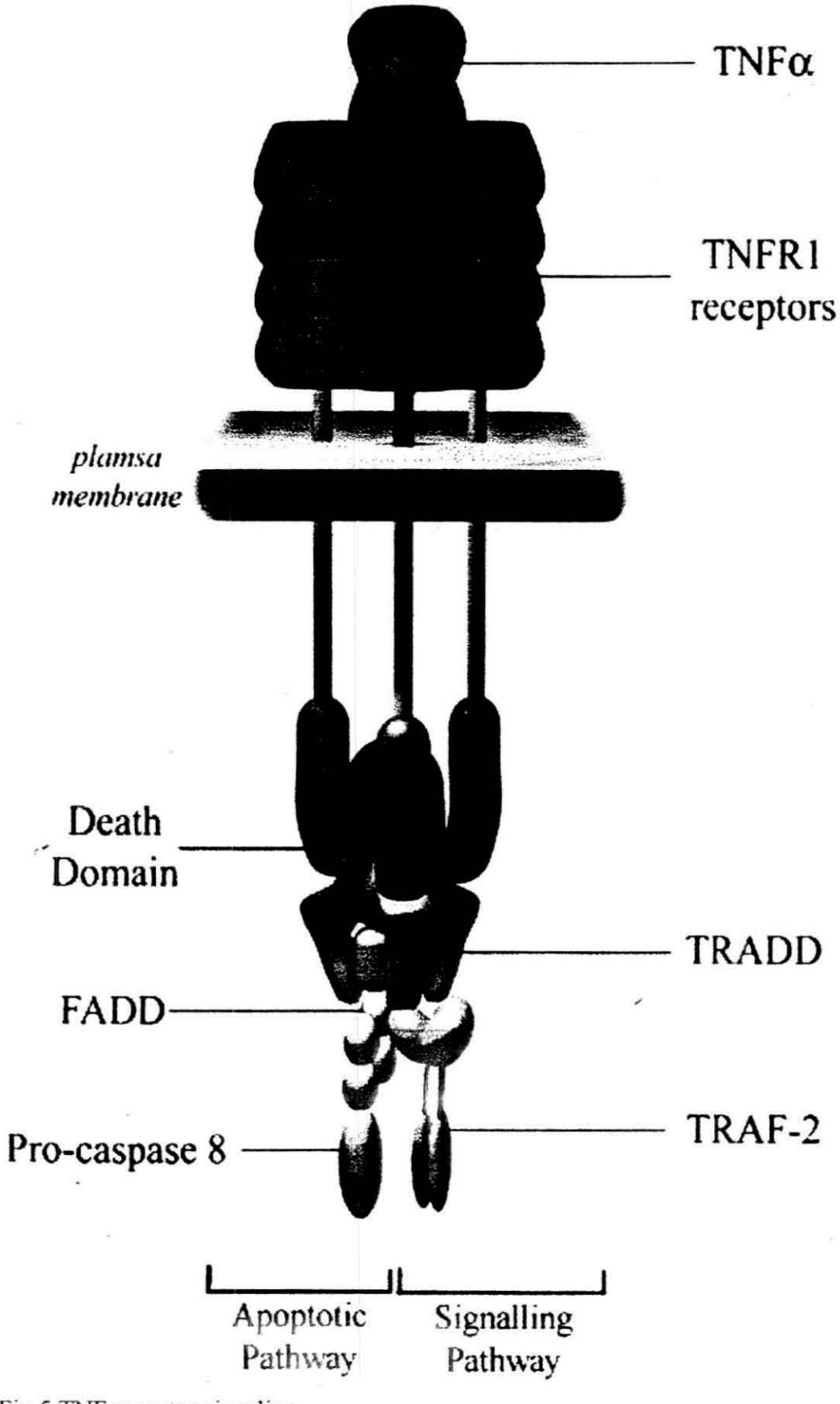

Fig.5 TNF receptor signaling

Binding of TNF alpha to TNFR1 results in receptor trimerisation and clustering of intracellular death domains. This allows binding of an intracellular adapter molecule called TRADD (TNFR-associated death domain) via interactions between death domains. TRADD has the ability to recruit a number of different proteins to the activated receptor. Recruitment of TRAF2 (TNF-associated factor 2) leads to activation of NF-kB and the JNK/Ap-1 pathway.

TRADD can also associate with FADD, which leads to the induction of apoptosis via the recruitment and cleavage of pro-caspase 8. TNFR1 is also able to mediate apoptosis through the recruitment of an adapter molecule called RAIDD (RIP-associated ICH-1 / CED-3 homologous protein with a death domain). RAIDD associates with RIP through interactions between death domains and can recruit caspase 2 through an interaction with a motif, similar to the death effector domain, known as CARD (caspase recruitment domain). Recruitment of caspase 2 leads to induction of apoptosis.

Signaling by CD95 / Fas

There are three main roles of CD95:

1. Cytotoxic T-cell mediated killing of cells (for example, CTL-mediated killing of virus-infected cells)
2. Deletion of activated T-cells at the end of an immune response
3. Destruction of inflammatory and immune cells in immune-privileged sites

The activation of apoptosis through CD95/Fas signaling is shown in figure. The ligand for CD95 (CD95L or FasL) is a trimer that on association with the receptor promotes receptor trimerisation that in turns results in intracellular clustering of parts of the receptor called death domains (DD). This allows an adapter protein called FADD (Fas-associated death domain) to associate with the receptor through an interaction between homologous death domains on the receptor and on FADD. As well as containing a death domain, FADD also contains a death effector domain (DED). The death effector domain allows binding of pro-caspase 8 to the CD95-FADD complex. Pro-caspase 8 (also known as FLICE) associates with FADD though its own death effector domain, and upon recruitment by FADD is immediately cleaved to produce caspase 8. This then triggers activation of execution caspases such as caspase 9. The complex of proteins – CD95, FADD and pro-caspase 8 – that trigger apoptosis is also known as DISC or Death Inducing Signaling Complex.

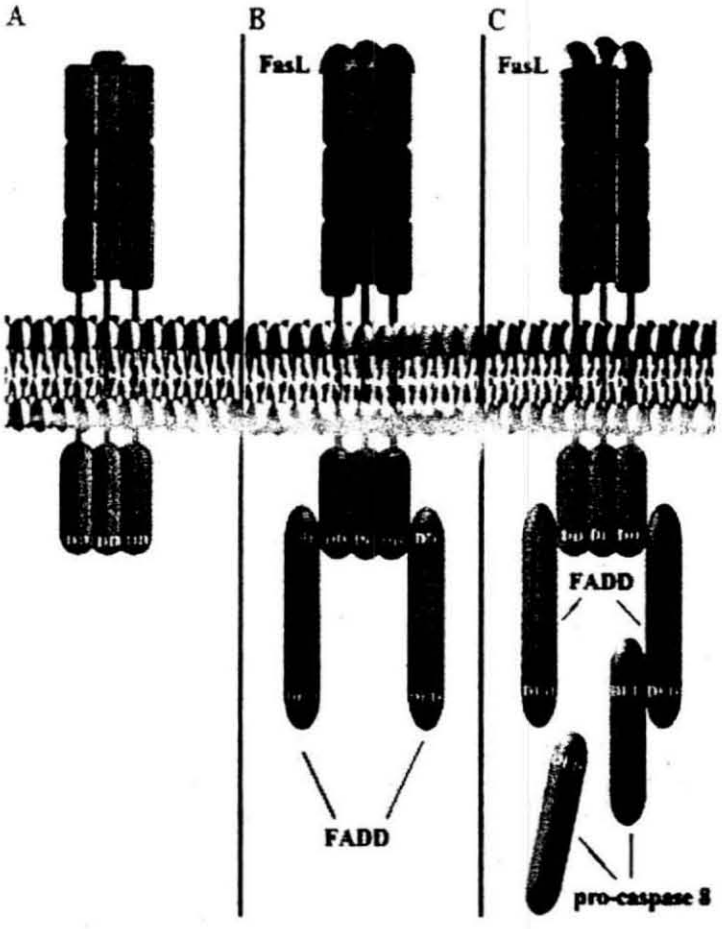

Fig.6 Activation of apoptosis through CD95 / Fas

Induction of apoptosis by TRAIL

In a number of ways TRAIL (TNF-related apoptosis inducing ligand) is similar in action to CD95. Binding of TRAIL to its receptors DR4 or DR5 triggers rapid apoptosis in many cells, however unlike CD95, its expression has been shown to be constitutive in many tissues. The DR4 and DR5 receptors contain death domains in their intracellular domain, but as yet no adapter molecule (such as FADD or TRADD) has been identified that associates with the receptor to initiate apoptosis. Work in FADD-deficient mice has indicated that FADD is not essential for triggering apoptosis via these receptors.

Since DR4 and DR5 mRNA has been shown to be expressed constitutively in several tissues, it has been suggested that there are mechanisms that protect cells from apoptosis. One possible mechanism of protection is based on a set of decoy receptors that compete for binding of TRAIL with the DR4 and DR5 receptors. The decoy receptors are called DcR1 and DcR2. Both of these receptors are capable of competing with DR4 or DR5 receptors for

binding to the ligand (TRAIL), however ligation of these receptors does not initiate apoptosis since DcR1 does not possess a cytoplasmic domain, while DcR2 has a truncated death domain lacking 4 out of 6 amino acids essential for recruiting adapter proteins.

Bcl-2 proteins

The bcl-2 proteins are a family of proteins involved in the response to apoptosis. Some of these proteins (such as bcl-2 and bcl-XL) are anti-apoptotic, while others (such as Bad or Bax) are pro-apoptotic. The sensitivity of cells to apoptotic stimuli can depend on the balance of pro- and anti-apoptotic bcl-2 proteins. When there is an excess of pro-apoptotic proteins the cells are more sensitive to apoptosis, when there is an excess of anti-apoptotic proteins the cells will tend to be less sensitive.

The pro-apoptotic bcl-2 proteins are often found in the cytosol where the act as sensors of cellular damage or stress. Following cellular stress they relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. This interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic bcl-2 proteins and can lead to the formation of pores in the mitochondria and the release of cytochrome C and other pro-apoptotic molecules from the intermembrane space. This in turn leads to the formation of the apoptosome and the activation of the caspase cascade.

RAPID METHODS FOR DIAGNOSIS OF FISH AND SHELLFISH PATHOGENS

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Introduction

In order to prevent disease outbreaks, minimize the presence of pathogens and to reduce the dependence on antimicrobial compounds, rapid diagnostic steps are essential. While considering rapid diagnostic tests, it should be ensured that they fulfill the following criteria:

1. Compared to other procedures, they should be more speedy, sensitive and accurate.
2. The tests should have presumptive and/or confirmatory application.
3. The tests be micro-modified or automated and inexpensive handling of large number of individuals and small volume samples.
4. The tests should require non-destructive samples.
5. The tests must yield qualitative and quantitative results.
6. The results obtained from such tests should correlate with the other clinical symptoms of the fish.

IMMUNOASSAY DIAGNOSTIC METHODS:

Polyclonal versus Monoclonal antibodies:

Immunoassays take advantage of the natural specificity of antibodies toward foreign objects. The immunoassays can utilize polyclonal or monoclonal antibodies in a variety of formats to provide rapid detection of infectious agents. Among these, monoclonal antibodies are favored due to their high degree of specificity. The method of production of monoclonal antibodies is as follows:

1. Selection of cell lines to be fused
2. Somatic cell fusion
3. Selection of hybrid cells
4. Selection desired clones
5. Production of antibodies from selected clones

DIRECT FLUORESCENT ANTIBODY TEST (D-FAT):

The D-FAT procedure has gained widespread use in finfish culture. It uses antibody prepared against the pathogen of interest and then conjugate it with a fluorescing dye (Fluorochorme). An impression smear, bacterial culture, tissue culture showing cytopathic effect (CPE) or specially prepared tissue sections on a microscope slide can be examined for specific pathogens using fluorescently labeled antibodies. Wherever, the antibodies are attaching to its target, the target glows when viewed through the fluorescence microscope.

Though this technique has the advantage of visually pin pointing the pathogen, and its location within the tissue, it requires the use of an expensive fluorescence microscope and suffers from expertise (subjective) interpretation of the results.

ENZYME-LINKED IMMUNOSORBANT ASSAY (ELISA)

Enzyme immune assays (EIA s) are most widely used as antibody-based diagnostic technique. In these techniques, the antibody molecules are linked to enzymes either directly or indirectly. In the direct method, the enzyme is conjugated (linked) to a portion of the antibody molecule that recognizes the antigen. This is the "lock and key". In the indirect method, a second step is required. Here, a carrier or second antibody is linked with the enzyme. The amount of enzyme is important in producing measurable signal when the primary antibody binds to its target. The antibody is applied to tissue sections on microscope slides. This permits the antigen for pinpointing within a tissue using a normal light microscope. Apart from the diagnostic applications, this technique also helps in studying how the pathogen spreads within the organism and causes disease.

The ELISA detects specific substances in a complex mixture by binding them to antigen or antibody-coated substances. It is also capable of detecting viruses, bacteria, drugs, hormones, toxins and carcinogens, depending on the nature of ELISA. Once binding has occurred, other reagents are added that allow the captured substances to be linked to indicators or enzymes, which can be quantified. An example of working principle of ELISA in the case of shrimp pathogenic *Vibrio* species is summarized below:

1. Raise antibody against pathogenic *Vibrio* in goat or rabbit (inject the purified antigen fraction of bacteria into rabbits).

⇓

2. Take hepatopancreas / body tissue sample - mix with phosphate-buffered saline (PBS) pH 7.4 + 0.05% tween 20 (PBS - T 20). Heat at 100° C for 15 minutes.

⇓

3. Purified rabbit immunoglobulin containing antibodies against antigens of *Vibrio* is attached to the surface of 96-well microtiter plate. (Fig 1.)

⇓

4. Attachment is accomplished by diluting the antibody to the prescribed concentration in carbonate-bicarbonate buffer (pH 9.6) and incubating the plate overnight adding 200µl to each plate (16 h at 4°C).

⇓

5. Coating buffer or any unused antibody are removed by washing the wells with buffer.

⇓

6. Supernatants of centrifuged test samples are then applied in duplicate to the antibody-sensitized wells. Each well will receive 0.2ml so that the final test sample can be within 0.5 ml.

⇓

7. Labelling the plate is important. Usually the following controls are needed:

   a. A blank (B: Test for background with reagent only- no sample)
   b. Conjugate Control - (CC: For conjugate striking directly to plate)
   c. Substrate chromogen control (SC: Test for non-specific color development with substrate and chromogen only)
   d. Positive Control (1:100, 1:1000, 1:2000 and 1:5000 K&P)
   e. Negative Control: Appropriate tissue from control or uninfected fish/shellfish

⇓

8. Plates to be read at 405 nm.

The degree of color change is proportional to the amount of antigen in the sample, i.e., the wells containing samples from uninfected animals will not show any color whereas the others will show varying amounts of color change, which can provide quantitative analysis.

A few of the ELISA protocols are given in Table 1.

DNA-BASED DIAGNOSTICS:

The cloning and manipulating of genetic material has led to the development of extremely sensitive and specific diagnostic systems. For example, DNA based test formats have entered into the area of infectious disease diagnosis for aquatic species. The DiaXotics Inc, Wilton, CT are pioneers in commercializing DNA-based diagnostics. They produced 'Shrimp Probe' for detecting viral infections of shrimp.

The DNA probe is created by purifying the infectious agent of interest and isolating its nucleic acid. An exact copy of the DNA or a portion of the DNA is made by the cloning process. This copy or probe will bind to the original DNA of the pathogen whenever the two come into contact. In order to accomplish this efficiently, the DNA strands of both the pathogen and the probe must first be separated by heating. After the strands have been separated, one of the strands of the probe can bind to its complementary strand from the pathogen. By attaching a non-radioactive reporter molecule, such as digoxigenin (DIG), the hybrid DNA can be identified and measured (Reddington and Lightner, 1994).

POLYMERASE CHAIN REACTION (PCR)

In the Polymerase Chain Reaction, the DNA to be amplified is denatured by heating the sample in the presence of DNA polymerase and excess dNTPs, the oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. A product of

indeterminate length characterizes the first cycle; however, the second cycle produces the discrete "short product" which accumulates exponentially with each successive round of amplification. This can lead to many million-fold amplification of the discrete fragment over the course of 20 to 30 cycles. For example 30 cycles can result in 2 X 28 fold (270 million fold) amplification of the discrete product.

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Table 1. ELISA protocols

ELISA Protocol	Uses	Required reagents	Comments
Indirect	Antibody screening; epitope mapping	Antigen, pure or semipure: test solution containing antibody; enzyme conjugate that binds Ig of immunized species	Does not require the use of preexisting specific antibodies; requires relatively large amounts of antigen.
Direct competitive	Antigen screening; detect soluble antigen	Antigen, pure or semipure; test solution containing antigen; enzyme-antibody conjugate specific for antigen	Rapid assay with only two steps; excellent for measuring antigenic cross reactivity
Antibody-sandwich	Antigen screening; detect soluble antigen	Capture antibody (purified or semi-purifeid specific antibody); test solution containing antigen; enzyme-antibody conjugated specific for antigen	Most sensitive antigen assay; requires relatively large amount of pure or semi-pure specific antibody (capture antibody)
Double antibody-sandwich	Antibody-screening epitope mapping	Capture antibody: (specific for Ig of immunized species); test solution containing antigen; enzyme-antibody conjugate specific for antigen	Does not require purified antigen; relatively long assay with five steps
Direct cellular	Screen cells for expression of antigen; measure cellular antigen expression	Cells that express antigen of interest; enzyme-antibody conjugate specific for cellular antigen	Sensitive assay for bulk screening; insensitive to heterogeneity of expression in mixed population of cells
Indirect cellular	Screen for antibodies against cellular antigens	Cells used for immunizing; test solution containing antibodies; enzyme conjugate that binds Ig of immunized species.	May not detect antibodies specific for cellular antigens expressed at a low density

PCR BASED RAPID DIAGNOSIS OF PATHOGENS

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Introduction

Use of PCR assisted diagnosis has many advantages over the conventional methods. Since PCR can amplify oven a single strand of DNA into millions of copies within hours, even a single bacterial cell collected from the infected fish could be used directly for the identification. Thus considerable time can be saved.

Two of the PCR assisted diagnostic techniques merits special mention. They are the DNA fingerprinting based an arbitrarily primed PCR (AP PCR) and amplification of species specific virulence gene using specific primers flanking these genes. Before going to the details of these techniques let us examine how the PCR work

The Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) is a technique for the *in vitro* synthesis billions of copies of a specific nucleic acid sequence by performing successive rounds of *in vitro* nucleic acid replication. This is achieved by using two oligonucleotide primers that hybridize (annealing) to the opposite strand of the target DNA at positions that flank the region to be amplified through simultaneous extension of both primers. A repetitive series of cycles involving template denaturation, primer annealing and extension of the annealed primers by DNA polymerase results in the exponential accumulation of the DNA whose termini are defined by the 5' ends of the primers. Since the primer extension products synthesised in one cycle can serve a template for the next, the number of target DNA copies approximately doubles at every cycle. Thus 20 cycles of PCR, yields about a million fold amplification.

The standard PCR mixture in addition to the sample (template) DNA contains 50mM KCl, 10 mM Tris HCl (pH 8.4), 1.5 mM $MgCl_2$, 100 μg/ml gelatin, 0.23 μm of each primer, 200 μm of each deoxyribonucleoside triphosphate (dATP, dCTP, dGTP and dTTP) and 2.5 units of Taq polymerase. The sample DNA generally contains 10^2 to 10^5 copies of template. The volume is made up to 25 or 50μl. The amplification is performed in a DNA thermal cycler, each cycle consisting of denaturation at 94°C for 30- 50 sec, annealing at 55°C for 30- 90 sec and extension at 72°C for 60- 120 sec for total of 30 cycles. Cycling could include a final extension at 72°C for 5 min. Reactions are stopped by chilling at 4°C or by addition of EDTA at 10mM.

Identification of pathogens through PCR amplification of species specific genes

All the pathogens possess certain genes mainly related to its virulance factors or toxins which are specific to that pathogen. The core sequence of these genes are usually highly conserved. Therefore, an ideal approach for the identification of the suspected pathogen is to amplify such genes through PCR, using primers synthesized to have complementarity to the conserved regions of these genes. This implies that , previous knowledge of the sequence of the species specific gene should be available. With the increasing availability of information on the sequences of such genes of the common bacterial pathogens, application of this method shall become more common.

Given below is the list of specific genes of certain bacterial species that could be utilized for identification

Bacterial Species	Gene
Vibrio vulnificus	cth (Cytotoxin haemolysin gene)
Vibrio cholera	ctxA (Cholera toxin gene) ctxB
V. parahaemolyticus (K+)	tdh (Thermostable direct haemoysin gene)
Listeria monocytogene	dth (Delayed hypersensitivity factor) Iap (Invasion associated protein gene) in1A (Internalin protein gene)
Yersina enterocolitica	YadA (Virulence associated outer membrane protein)
Luminous bacteria	LuxA (Luciferase gene) Lux B

The potential application of these genes for the identification of the bacterial species have been demonstrated by different workers. For example a 300 bp fragment of dth-18 gene of *Listeria monocytogenes* amplified by Fluit *et al.* (1993) using two20 bp primers viz;

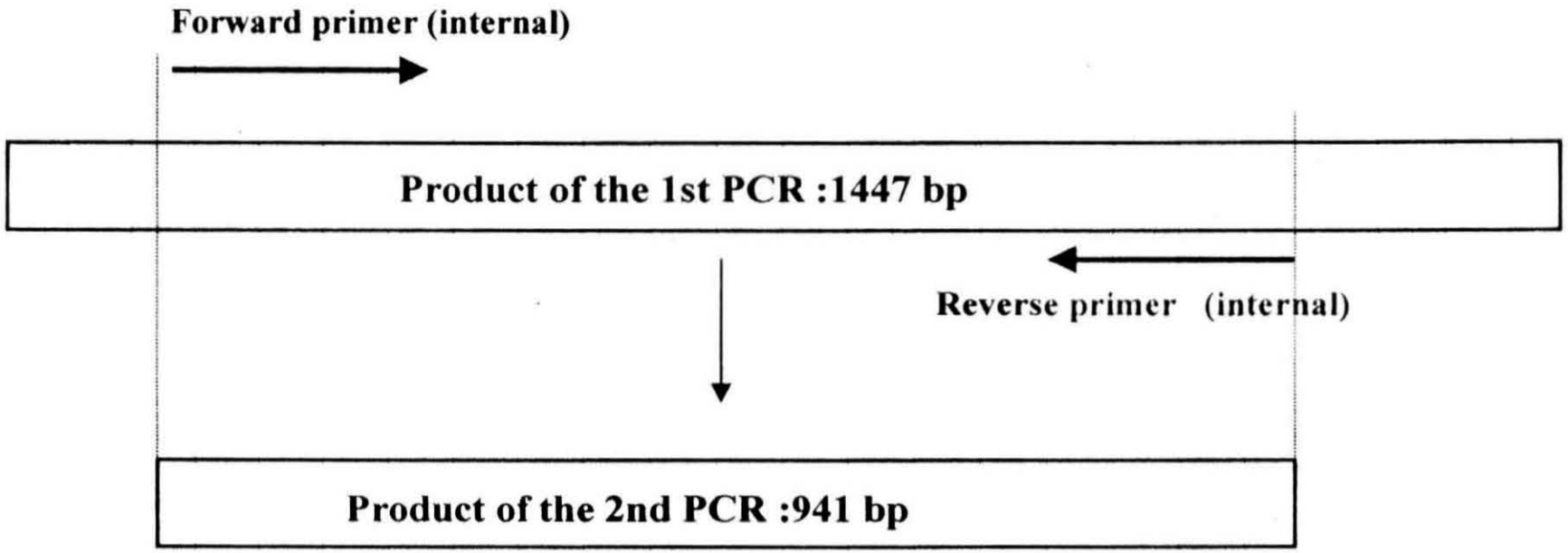
5'CTA ATC AAG ACA ATA AAA TC and

5'GTT AGT TCT ACA TCA CCT GA

was found to be useful for identifying L.monocytogenes. Similarly, a 564 bp fragment of the ctxA gene of *Vibrio cholerae* amplified by Fields *et al.,* (1992) using two primers of 21 bp each, was claimed to be useful in identifying *V. cholerae* bacteria.

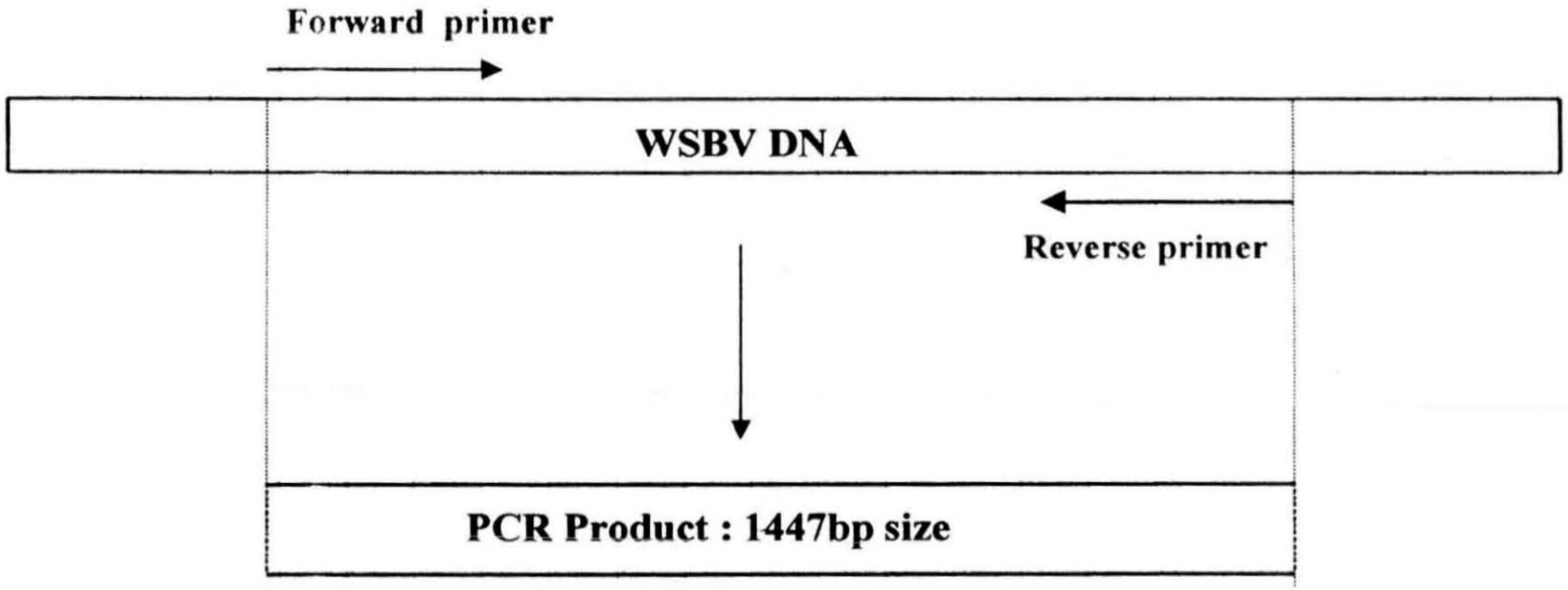
Nested PCR of W S S V

(Primers based on *Sal* I 1461bp segment of WSBV)

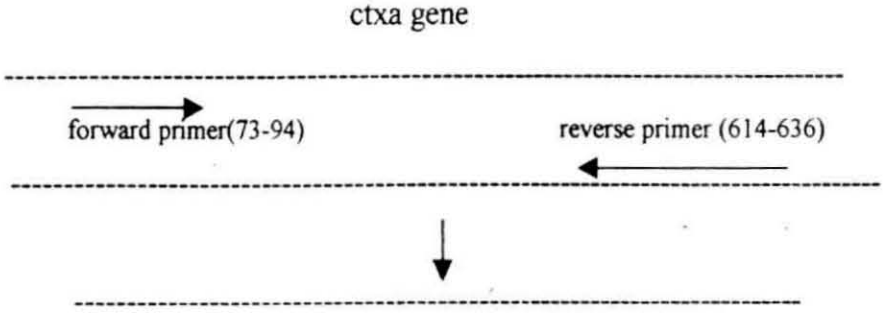


PCR amplification WSSV

(Primers based on *Sal* I 1461bp segment of WSSV)



PCR amplification of ctxA gene segment of *V. Cholerae*



(Expected PCR product) 564bp

The sensitivity and specificity of the PCR can be further enhanced by carrying out nested PCR. The process utilizes two consecutive PCRs. The first PCR utilizes a pair of primers flanking the gene in question while the second PCR uses another pair of primers having complementarity to an internal segment of the gene which was amplified in the first PCR. The larger fragment produced by the first reaction is used as the template for the second PCR. Therefore, when information on the sequence of species specific genes are available, amplification and visualization of that gene using a nested PCR is the method of choice when viewed from the point of sensitivity and reliability.

Detection of White Spot Syndrome Virus

White spot disease caused by White spot syndrome (WSSV) is one of the major threat faced by the shrimp farming industry. As there is no cure for this disease, stocking diséase free larvae is one of the management measures recommended to prevent critical transmission of the virus.

Till recently, the diagnostic technique were largely dependent upon history of disease, clinical signs and histological examination of moribund animals. From the practical point of view, these procedures are of limited use in disease prevention and sensitivity is often limited.

Of late, biotechnological diagnostic techniques using DNA probes, PCR amplification of conserved DNA sequence using specific primers are being applied to detect the presence of virus in the animal even in the latent form. These tests are highly specific and highly sensitive.

DNA probes could be used to detect the virus in clinically infected animals either using tissue extracts or through *in situ* hybridization of shrimp tissue sections. The virus can be clearly localized in the shrimp sections. Strong labeling is limited to infected nuclei and is strong only when there is hypertrophy of the nucleus. When infection is low, histological diagnosis is particularly difficult. In contrast, diagnosis with gene probes is more specific and

sensitive and it could be used as a diagnostic tool for WSBV infection using haemolymph samples from live shrimp.

PCR Screening of White Spot syndrome Virus

The infection can be either vertical transmission ie, from parent to the larvae or horizontal transmission i.e., from animal to animal. Hence, stocking of disease free larvae produced by disease free parents is of the primary requirement in the prevention and control of disease. Thus, use of very sensitive molecular diagnostic tests which can detect very low levels and latent forms is very essential. PCR amplification of single copy gene of the virus is a test which can meet these requirements. Among the various kinds of molecular diagnostic kits being marketed for White spot disease virus detection, the PCR based detection is the most sensitive and very useful for the pro-active disease management. This is much more sensitive than the nucleic acid probes. While the sensitivity of most probes is around 10^4 - 10^5 molecules of a homologous target, on an average less than 10 target molecules are sufficient to provide a positive result by PCR based test. PCR based method can be used to detect WSSV in brood stock larvae and other carrier animals. Stocking only WSSV negative larvae in culture system is the essential step in preventing vertical transmission of the disease into the cultured shrimps.

During the culture period monitor shrimp health with PCR screening. It is necessary to monitor the disease status routinely. Samples should be collected every fortnight and sent to a reliable laboratory for PCR screening. The PCR technique will detect early infection and enable the farmers to adapt a suitable strategy to minimize losses. Diagnosis relying only on the appearance of white spots does not help as by then the shrimps will die within a few days resulting in severe losses.

Duplex PCR Screening of White Spot disease Virus

CMFRI has designed a duplex PCR for detection of WSSV which is cost effective, faster and reliable compared to the nested PCR kits being used currently. This involves the simultaneous PCR screening of different regions of the viral genome with appropriate primers

The duplex PCR has the following advantages :

- Rapidity : While nested PCR is carried out in two stages, duplex PCR is conducted in a single run, thus, reducing time required for the screening.

- Cost effectiveness : Since the assay volume and constituents used in the duplex PCR is equivalent to that of a single run of the nested PCR, it is less expensive.

- Reliability : Since different regions of the viral genome are amplified and checked simultaneously , it has got high reliability also.

Suggested Readings

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ISOLATION AND APPLICATION OF MARINE NATURAL PRODUCTS

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Introduction

The marine bioactive compounds or Marine natural products (MNPs) offer avenues for developing cost-effective, safe and potent novel drugs and other useful products. MNPs are organic compounds produced by microbes, sponges, seaweeds, and other marine organisms. The host organism biosynthesizes these compounds as non-primary or secondary metabolites to protect themselves and to maintain homeostasis in their environment. In the decade from 1977 to 1987, around 2500 new metabolites (MNPs) were reported from marine organisms ranging from microbes to fish. According to Dr. S.Z. Qasim, less than 1.0% of the total marine organisms have only been examined for MNPs research. Perusal of literature indicated that even the seawater has bactericidal properties. This could be attributed to the production of antibiotics by planktonic algae and bacteria respectively. Considering such vast resource, holistic approach of pharmacological research has to be given to develop potent therapeutics.

Marine bacteria

The discovery of penicillin in 1929 heralded the era of antibiotics, which led to the understanding that microorganisms are a rich source of useful natural products. Since that time and consequent to the continuous effort of R&D projects with heavy investments, nearly 50,000 natural products have been discovered from microorganisms. Of these substances, more than 10,000 are biologically active and more than 8000 are antibiotics. More than 100 microbial products are in use today as antibiotics, anti-tumor agents and agrochemicals. It is demonstrated that marine bacteria produce anti-microbial substances. Further array of research results indicated that the marine microorganisms are capable of producing potent and unusual bioactive natural products that are uncommon in terrestrial microbes.

The first documented identification of a bioactive marine bacterial metabolite was the highly brominated pyrrole antibiotic, isolated by Burkholder and co-workers from a bacterium obtained from the surface of the Caribbean Sea grass. Subsequently, this unique metabolite was identified by x-ray crystallographic methods, which composed of more than 70% bromine by weight. The metabolite exhibited impressive *in vitro* antibiotic properties against Gram-positive bacteria, with minimum inhibitory concentration (MIC) ranging from 0.0063 to 0.2 μg/ml. However, it was inactive for Gram-negative bacteria and animal assays.

As more evidence is obtained, it is becoming abundantly clear that bacteria form highly specific, symbiotic relationships with marine plants and animals. Experience in this area arose from a study of the pathogen resistance of the estuarine shrimp *Palaemon macrodactylus*. The eggs of *P. dactylus* possess significant bacterial epibionts, which, when removed by treatment with antibiotics, leads to the rapid infestation of the eggs by pathogenic fungi, especially of *Lagenidium callinectes.* Although there are many plausible mechanisms to explain this protective phenomenon, with *Palaemon* it could be anti-fungal agents produced by bacteria.

Marine fungi

Although terrestrial fungi have represented a major biomedicinal resource (penicillin from *Penicillium*, for example), studies to develop the biomedicinal potential of marine fungi have been few. The isolation of a small lactone, leptosphaerin from *Leptosphaeria oraemaris* by Schiehser in1980 demonstrated that marine fungi may form important resource for unique metabolites. Later, the useful chemical, Gliovictin was isolated from marine fungus, *Asteromyces cruciatus.* Since then more than twenty useful bioactive compounds have been derived from marine fungi.

Marine Micro algae

Micro algae are significant resource for bioactive metabolites, particularly cytotoxic agents with applications in cancer chemotherapy. From the marine micro algae such as from the blooms of *Phaeocystis* sp., antibiotic substances are reported. *Phaeocystis pouchetii* produces chemicals such as Acrylic acid, which constitutes about 7.0% of the dry weight. The antibiotic substances thus produced are transferred throughout the food chain and found in the digestive tract of Antarctic penguins. Production of β carotene and vitamins by the halo tolerant alga *Dunaliella sp.*, is documented.

Marine Macro algae

It is reported that of the total marine algae so far evaluated, 25% showed one or the other biological activity. Metabolites of green algae were reported to contain 1,4 – diacetoxic butadiene moiety, which exhibited icthyotoxic property. Antimicrobial diterpene (dictyodiol) was isolated from *Dictyota spinulosa*. Both icthyotoxic as well as cytotoxic diterpenoids from *Dictyota sp.* Among the red algae, halogenated lipids have been isolated, particularly from the *Laurencia sp.* The rare chemical prostaglandin was also reported to occur in *Gracilaria pichenoids*. *Ulva* meal supplementation was found to provide disease resistance to red sea bream in Japan. Similar results were also reported from Japan on the use of *Ulva* meal supplementation towards disease resistance and high growth rate in black sea bream. The polysaccharide fractions from marine algae, *Porphyra yezoensis* (PASF) was found to stimulate the *in vivo* and *in vitro* murine phagocytic function. The purified fractions of PASF gave stronger phagocytic activity.

An antiviral compound, UF-131 was isolated from the green alga *Ulva fasciata*. Extracts of *Caulerpa taxifolia* and *Ulva fasciata* with moderate antiviral activity was found to protect the animals. Among the 30 different species of algae collected and screened for activity biological activity was recorded in 26 species towards bacteria. Growth of gram-negative bacteria such as *Vibrio* and *Staphylococcus* and gram-positive bacteria such as *Bacillus sp* were found to be inhibited to varying degrees. Several species of *Halinida* produce terpenic di or tri aldehydes with icthyotoxic activity. In addition, brominated aromatics, nitrogen heterocycles, nitrogen-sulfur heterocycle, sterols, terpenes, dibutenoloides, protein, peptides and sulfated polysaccharides were also reported. The sulphated polysaccharides grouped as carrageenens and agar isolated from red and brown algae were shown to possess antigenic properties and exhibited anticoagulant and antiviral activities. Other compounds from algae were shown to possess antifungal, antibacterial, icthyotoxic, hypotensive and antihelminthic activities.

Under the ICAR Ad-hoc scheme sanctioned by the Council, basic data on the availability of different algae along the coasts of India with their secondary metabolite capabilities were carried out in CMFRI. Seventeen potential macro algal extracts were prepared and their bioactivity profiles were studied. Among the macro algal crude extracts, experiments conducted with pathogenic challenge among fish and shellfish indicated their potential therapeutic nature. The responses of the fish/shellfish towards treatment with a few of the algal metabolites have been also standardized in CMFRI laboratory.

Marine Sponges

In sponges, the secondary metabolites are synthesized to protect themselves and to maintain homeostasis. The wider biosynthetic capability of sponges could be attributed to their biological association with other symbionts. According to Bertrand and Vacelet, about 38% of the sponge body comprises of microorganisms. A wide variety of secondary metabolites were isolated from sponges and these have been associated with antibacterial, antimicrobial, antiviral, antifouling, HIV-protease inhibitory, HIV reverse transcriptase inhibitory, immuno- suppressent and cytotoxic activities. In addition to potential anticancer applications, the MNPs of sponges have a myriad of activities ranging from antibiotic activity including anticoagulant, antithrombin, anti-inflammatory, as well as imunomodulatory activities.

During the 1970s and 1980s, investigators also studied the type and specificity of sponge-microbe associations, often using microscopy to show the presence of specific symbiont morphologies within specific sponges. The molecular basis of symbiosis was also probed, albeit to a lesser extent. Bacteria, which live symbiotically with sponges, can be passed through their feeding chambers without being digested. This suggested some sort of encapsulation or recognition process. In the demosponge *Halichondria panicea,* an

association with the microbe *Pseudomonas insolita* may be lectin-based. An immunological basis for symbiosis in some sponges is claimed as evidence of a Precambrian origin for many symbioses.

A major problem with the early studies on sponge-microbe symbiosis was that most microorganisms were uncultured or uncultivable, so descriptions of symbioses usually relied either on morphology of symbionts or chemical measurements of nutrient transfer. Even in the cases where putative symbionts could be cultured, the ecological relevance of symbiosis could not be determined. The period following Wilkinson's review has been marked by the ascendance of molecular biological techniques in environmental microbiology, which have allowed investigators to focus on uncultured microorganisms.

The application of molecular biology to sponge-microbe symbiosis is yielding results that could not have been obtained by classical microbiological methods. The discovery of a member of the Archaea living specifically within a sponge similar to *Axinella mexicana* was a particularly exciting. The archaeal microorganism, *Cenarchaeum symbiosum* (P: Crenarchaeota), lives at a relatively cold 10 °C and is therefore considered sychrophilic. Subsequent *in situ* hybridization experiments showed which microorganism in the sponge was archaeal and allowed localization of the symbiont.

Unfortunately, despite the scientific and technological benefits of determining the source organisms of interesting and bioactive metabolites, a microbial origin of sponge compounds has rarely been demonstrated. Most of the literature is purely speculative, based on similarities, however slight, between compounds from sponges and those from cultivated microorganisms, especially cyanobacteria. Several researchers have attempted to culture microorganisms from invertebrates in the hopes of obtaining some of these bioactive compounds. Although they have been successful in the discovery of novel natural products, this research has rarely demonstrated the presence of sponge metabolites in the microbial isolates. In one case the same compound was found in a *Hyatella* sp. sponge as in a *Vibrio* sp. cultured from that sponge. These results demonstrate that traditional culturing approaches are not generally applicable to the environmental problems of sponge-microbe symbiosis. Two cases of symbiont production of sponge compounds have been clearly established. Both studies relied on cell fixation and physical separation techniques, bypassing the problem of culturing symbiotic microorganisms. Unson separated cyanobacterial symbionts from the sponge *Dysidea herbacea* by flow cytometry and showed that chlorinated amino acid derivatives could only be found in the cyanobacterial fraction, while terpenes were localized in the sponge cell fraction.

Sponges contain a standing population of bacteria inside; in some instances, the bacterial load may come up to 38% of the total volume of sponge. The bacterial population is seen even in the motile larval stages. How and when this bacterial population gets into the larvae of sponges in not known. It is believed that the sponge phagocytoses these bacteria when food becomes scarce. It is not sure where the various bioactive substances in

sponges are produced by the sponge itself or by the associate or through the combined action of both.

Avenues for further research and development:

Perusal of literature indicates that during the last three decades number of diverse biologically active compounds has been isolated from marine organisms, but the number of compounds taken-up for the field trial/clinical use are scanty. This may be due to the failure of successful collection of concerned source organism in bulk, which have same sort of secondary metabolites. Some of the future requirements are listed below:

Microbial Isolation/screening and culture techniques:

As the symbiotic microbes are difficult to culture under laboratory conditions, basic Research in Marine Microbiology is essential. Without considerable attention to developing the basic biology of marine microorganisms, explorations for new bioactive metabolites would be limited to those few classes of microorganisms, which are readily isolated and grown under "standard" conditions. Unfortunately, little is known about the specific nutrients and growth factors required by most of the marine microbes. For example, the common media components such as peptone, sugars etc., are unrealistic marine nutrients as complex carbon sources such as chitin, sulphated polysaccharides, marine protein etc., are found in the marine habitat. In addition, information is lacking on some of the uncommon inorganic elements such as lithium, silicon etc., abundant in the marine sediments. As a result of these difficulties, it is seen that less than 5% of the available microbial population is only cultivable under the standard laboratory conditions. Presently, this condition, certainly limits the scope and ability to isolate and culture majority of the interesting and new microbes.

Preparation of crude extracts for bioactivity:

As the goal is to obtain the widest possible screening for each crude extract so that no useful compound is over looked. Solvents such as methanol, chloroform or ether as independent solvents or as combinations can be used depending upon the nature of the MNPs. As soon as the crude extracts are obtained, there is need for immediate and simple *in vitro* assays such as: i. Antimicrobial and ii. Enzyme inhibition assay (very low quantity of sample only is required). This in turn helps in the 'bioassay – guided fractionation and purification' process.

Purification:

Once bioactivity is detected in the crude extract, the next step is to purify the same. It is important to employ non-destructive method such as spectroscopic method, which conserves the materials for further bioassays. In addition, techniques such as: TLC, MS/IR/uv and H nMR – (for structural elucidations) are to be adopted for purification of the crude extract and for determining the structure.

Pharmacological screening:

The next step after purification and structural elucidation is pharmacological screening. Studies such as determining the LD_{50} of the extracts in mice, in addition to brine shrimp assay, fertilized sea urchin assay and starfish assay are to be carried out in established laboratories. Further tests such as: antiviral (AIDS/anti-HIV), cytotoxic, anti-inflammatory, anti-tumor, tumor promoter (protein kinase), analgesic, anti-coagulant / anti-thrombic (ex: heparin), anti-ulcer, anti-cholesterol / anti-lipemic, wound dressing, anti-parasitic, anti-protozoa are to be conducted.

Commercial development of bioactive (MNP) products:

The `co-operative drug development programme' as suggested by Faulkner (1993) is the best method, which will solve the problems arising on issues such as: patent rights, academic freedom and industrial secrecy.

Conservational aspects of source organisms:

Eco-friendly collection of the source organism and required supply of them in bulk for scaling-up process.

The role of Industry and Academia:

Considering the less microbiological and intensive pharmacological training to the industrial personnel, relevant microbiological training has to be imparted to the industrial pharmacologists. New isolation methods, media development etc., are to be included in the curricula of academic/research institutes. Collaborative programmes which combine biomedical and microbiological expertise of the pharmaceutical industry with the marine resources available in the marine R&D Institutes will in the long run help in the better utilization of the marine organisms or resources for biotechnological aspects.

DEVELOPMENTAL PROCESS IN MARINE PHARMACEUTICALS

PRIMARY ISOLATION

SPECIFIC ACTIVITY SCREENING
Unique or potentially useful biologicals

SOURCE MATERIALS
Bulk collection of source materials to isolate characterize the active compounds

IN VIVO ACTIVITY SCREENING
Tests to determine relative toxicity ant activity in animals

PURIFICATION AND CHARCTERISATION
Bioassay guided purification and structural elucidation

PHARMACOLOGICAL STUDIES
Testing for drug activity and safety

SCALING-UP PROCESS
Chemical or Biosynthesis

GOVERNMENTAL APPROVAL
Disclosure of complete drug source and manufacturing information,
preclinical results and future research plans

CLINICAL EVALUATION
Testing with human volunteers to determine metabolism,
absorption, elimination, side effects, safe dose range
and method of administration.

Mollusc foot adherence assay

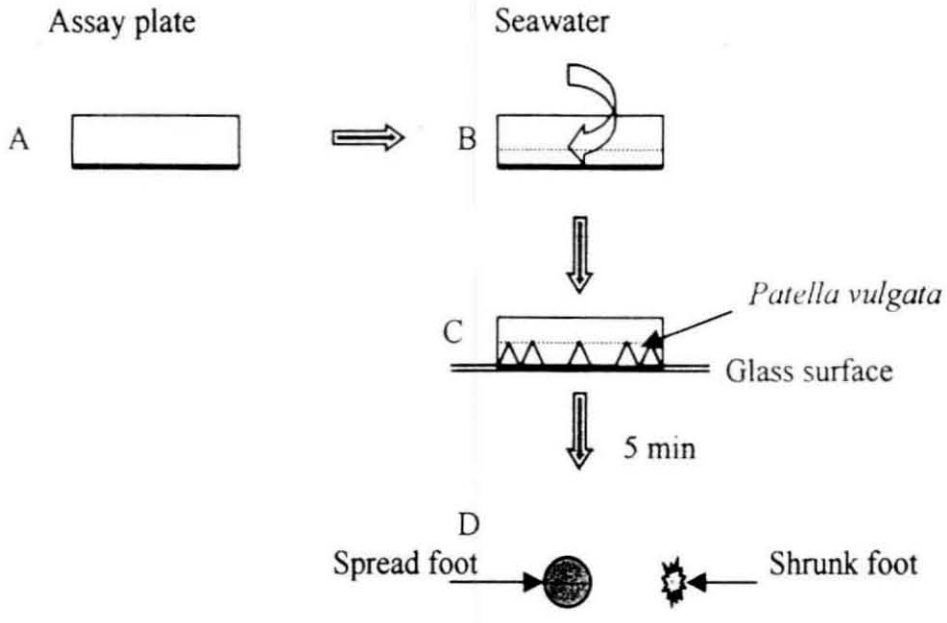

Figure 1. Procedure of the foot adherence assay:

A. Preparation of the assay plate with 1 ml of selected concentration of extract/fraction.

B. Filled with seawater without disturbance to the extract layer

C. The limpets were removed carefully from the tank and introduced in to the triplicate experimental plate and kept on an illuminated glass surface to observe the foot reflex.

D. Based on the foot adherence or shrinkage, the fouling rate can be estimated

PROPHYLACTIC MEASURES IN AQUACULTURE HEALTH MANAGEMENT

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Introduction

In any sphere of general health management, prevention is indeed better than cure. Bioremediation in aquaculture system is the basic biological process, which can be adopted with greater economic viability and without any environmental impairment for the health management of the cultured stock as a prophylactic measure. Apart from that, there are three more precise and powerful tools, which can be easily integrated into the aquaculture practice for health management. They are vaccines, immunostimulants and probiotics.

1. Vaccines

The concept of vaccination has been getting deep rooted in the aquaculture scenario over the years. Before examining the present status of vaccination, it would be appropriate to have glimpses of the immune system of fish and shellfishes.

Fish immune system

Finfishes have specific and non- - specific responses against pathogens. Non - specific responses occur prior to the activation of specific immune response which include agglutinins, lysins, complement, lysozyme, and the action of several non - specific phagocytic cells such as monocytes, granulocytes and thrombocytes and non - specific cytotoxic cells.

The specific immune system comprises two basic components called humoral and cellular immunity. The humoral immunity is carried out by B-lymphocytes where as cellular immunity is by the T lymphocytes. B cells produce antibodies or immunoglobulins, which are protein molecules that bind the foreign substances and antigens. These are then destroyed by various means like phagocytosis by macrophages. T cells do not produce antibodies by which they can recognize antigens by specialized receptors.

On primary exposure to an antigen, T and B cells cooperate in the response. A specific clone proliferates and matures into effector cells (B cells become plasma cells producing antibodies and T cells become cytotoxic or other effector cells or memory cells). This process constitutes the primary response. On subsequent stimulation by the same type of antigen, the memory cells again multiply in to effector cells or further in to memory cells, members of which then proliferate accounting for the stronger secondary response.

Inspite of recent research, there remains many gaps in our understanding of fish immune system. It is similar in many respects to that of mammals (e.g. Production of specific immunoglobulins, phagocytic or cytotoxic cells) but has its distinctive features (e.g. Reduced number and structural differences in immunoglobulin classes absence of lymph nodes etc.).

In case of immunization, the objective is to elicit a good response on challenge producing long lasting immunological memory, which is an effective and important factor than a strong and primary response.

Shellfish immune system

Based on the present understanding of shellfish immune system, it is believed that shrimp and other crustaceans do not possess adaptive immunity, having an innate system which include a diverse array of humoral and cellular factors such as phagocytosis, melanization by activation of the Prophenol oxidase activating system, clotting process, encapsulation of foreign materials, antimicrobial peptides and cell agglutination, non - self recognition factors such as lectins, lipoproteins, reactive oxygen intermediates (ROI), cytotoxicity and cell to cell communication.

The existing belief is that invertebrates lack specific immunoglobulins and so far no antibody molecules identical to those in vertebrates have been detected. However, several molecules identical to those in vertebrates have been detected in invertebrates. They include adhesion molecules such as catherins, Ig-like proteins, extracellular matrix proteins, trigrin, Limulus agglutination - aggregation factor (LAF), hemocytin, A74 protein, croquemort, plasmocyte spreading peptide (PSP1) and *Drosophila* Toll/ Cactus/ Dorsal Proteins (TCD). Apart from this, the presence of bactericidins in the haemolymph of *P. monodon* after exposure to heat killed *V. alginolyticus* has been reported and this would demonstrate some memory for different pathogen in the shrimp immune system.

Mode of preparation of fish vaccines

The bacterial fish vaccines may be categorized as follows:

1. Chemically or heat inactivated whole cells. These vaccines may be mono or polyvalent.
2. Inactivated soluble cell extracts. ie. Toxoids
3. Cell lysate
4. Attenuated live vaccines, possibly genetically engineered cells. There is a perceived risk that the vaccine strain may revert to pathogenic mode.

5. Purified sub-cellular components, e.g. LPS. These vaccines require a detailed understanding of microbial chemistry.

Eventhough it is difficult to identify any particular type of preparation, which excels in terms of protection. Generally, the simplest approach of using inactivated whole cells has received greatest attention. This technique was successful for a wide range of pathogens including *Aeromonas hydrophila*, *Edwardsiella ictaluri*, *Flavobacterium columnare*, *Photobacterium damsellae* subsp. *piscida*, *Vibrio anguillarum*, *V. ordalii*, *V. salmonicida* and *Yersinia ruckeri*. Whole cell vaccine gave superior results to other more complex forms of vaccines.

Methods of vaccine inactivation

There are seven methods of inactivating bacterial cells for incorporation into fish vaccines. These are the use of 3% v/v chloroform, 0.2-0.5% v/v formalin, 0.3-3.0% v/v phenol, heat (e.g. 56^0C or 100^0C), sonication and lysis with sodium hydroxide at pH 9.5 or with SDS. Commercially, the most interest has centred on use of formalin, which has given encouraging results.

Methods of administering vaccines to fish

A number of methods of administering vaccines to fish have been tried with varying degrees of success. They are:

1. Injection; 2. Oral uptake via food; 3. Immersion in a solution/suspension of the vaccine; 4. Bathing in a very dilute preparation of the vaccine for prolonged periods; 5. Spraying or showering the vaccine into fish; 6. Hyposmotic infiltration and 7. Anal intubation.

It is often difficult to determine which is the most effective method of vaccine application.

DNA vaccines (Gene vaccine)

DNA vaccines or gene vaccines are now [illegible] being investigated for use in a growing number of [illegible] including [illegible]. Here, DNA vaccine contains genes of disease agents that codes for one or more of the proteins used in traditional vaccines. The DNA vaccine is injected into muscle tissue cells of a susceptible host, which stimulates them to produce the proteins (antigens) that trigger immune system to recognize and attack an infection, by the real pathogen. Advantages of the DNA vaccines over traditional vaccine are1. Lower risk of accidental infection using live or attenuated pathogens; 2. DNA are more stable than proteins, making it more hardier during preparations, 3. DNA vaccines required to stimulate an immune response appear to be much lower than traditional vaccine. So far, three fish vaccines developed in collaboration with the Institute of Aquaculture, Stirling University, UK has been commercialized. They are vaccines against Vibriosis, Enteric Red

Mouth and Furunculosis. In India, efforts are under way to develop DNA vaccine against haemorrhagic septicemia caused by *Aeromonas hydrophila* in carps.

Biofilm vaccine

Among the various methods of fish vaccination, oral vaccination is most preferred by the farmers, as it is simple, free of stress and economical for mass application. However, oral vaccination usually elicits poor and inconsistent immune response and protection in fish, which is attributed to destruction of vaccine antigen in foregut and their failure to reach [illegible] explored for improving oral [illegible] microspheres, encapsulated antigens, enteric coated vaccines, adjuvants and bioencapsulation of vaccine in live feed. However, these methods are complex and costly for commercial aquaculture. In this background, biofilm form of a bacterial pathogen could be a better alternative oral vaccine. Bacterial biofilm is a colony of high density of cells embedded in a glycocalyx matrix on a substrate, which has been demonstrated to be resistant to action of antibiotics, chemical and host immune system. Therefore, the resistant nature of biofilm due to the protective glycocalyx coat and high density of cells could be ideally exploited for developing a resistant and effective oral vaccine for fish. A biofilm vaccine has been developed by College of Fisheries, Mangalore.

Vaccination in crustaceae

As the quest for understanding more about the non-specific defense mechanism of crustaceans has dominated research the prospects of developing vaccines to protect shrimp from specific pathogens such as WSSV were not seriously pursued. Nevertheless, effective vaccination of *Penaeus monodon* and *Penaeus japonicus* against Vibriosis with formalin killed *Vibrio* sp. had been carried out. It has been proved that vaccination against Vibriosis is effective up to even 50 days after vaccination and that the vaccinated group evidently had a higher survival rate than the glucan treated group, which they explained as the partial specificity of the vaccine-induced bactericidins. This prophylactic immunoprotection is accompanied by immune cell activation.

Previously, the presence of bactericidins in the haemolymph of *P. monodon* after exposure to heat killed *V. alginolyticus* had been reported, which demonstrated some memory for different pathogens in the shrimp immune system. Dealing directly with WSSV, an active accommodation of the virus resulting from an initial binding step by the host has been proposed, as a result of specific memory, such that simultaneous or subsequent viral binding for infection (by different receptors) did not trigger cellular apotopsis that lead to host death. Exposure of young larval stages in inactivated viral particles or sub unit viral proteins followed by subsequent challenge with active virus would result innocuous infections rather than mortality. A similar phenomenon was observed in a shrimp farm in Hiroshima, Japan against experimental PRDV (WSSV) challenge. Moreover, by artificial infection, an immune

shrimp was produced where resistance was confirmed by a viral re challenge and this phenomenon was explained as a quasi-immune response. Similar incidence have also been observed in different shrimp farms of Kerala where a culture could be completed successfully with a good harvest where an earlier viral attack had been reported. Centre for Fish Disease Diagnosis and Management, Cochin University of Science and Technology, has developed recently a WSSV vaccine preparation named as shrimpvac-1 and experimentally proved under laboratory and field conditions.

2. Immunostimulants

Immunostimulants are chemical compounds that activate the immune system of animals and render them more resistant to infection by virus, bacteria, fungi and parasites. It is known for many years that all wall fragments of microorganisms render animals more resistant to microbial infection. The ability of immune system to respond to microbial surface components is the result of an evolutionary process, where by animals have developed the mechanisms to detect common and highly conserved chemical structure of potentially pathogenic microorganisms and use those structures as "alarm signals" to switch on the defense against infection.

The immune system will respond to an immunostimulant as if challenged by a pathogenic microbe. Administration of an imunostimulant prior to an infection may thus protect against an otherwise severe to lethal infection.

Shrimps are primarily depending on non -specific immune processes for their resistance to infection. Increasing resistance to a specific pathogen may not always be a viable proposal and therefore, increasing the non-specific immunity of the shrimp to equip them with the broad-spectrum defensive ability is the appropriate strategy. Immunostimulants have shown to induce both immune related and non-immune related effects in shrimps. Immune related effects include enhanced phagocytic activity, activation of prophenol oxidase activity and increased clotting of haemolymph. Non - immune related effects include higher growth and survival, enhanced tolerance to water temperature, and higher tolerance to salinity and stress.

The important immunostimulants are the ones derived from 1. Yeast (Glucans or ...are available as products such as Macrogaurd, Betafecun, Curdlan, Krestin, Lentinan, Schizophyllan, Scleroglucan, Vita Stim Taito, SSG; 2. Bacteria, available in the form products such as Biostim, Picibanil (OK-432), Peptidoglycan, Bacillus Calmitte Guerin (BCG), FCA, Freunds complete adjuvant, lipopolysaccharide, Muramyl peptide; 3. Glycans from plants; 4. Chitin; 5. Peptides from the animal extracts such as thymopeptin, thymosin; 6. Synthetic compounds such as Bestatin lipopeptides, FK-565 and Levamisole.

Apart from being useful as immunostimulants, the above compounds and the preparation can be used as adjuvants in vaccine preaparation.

3. Probiotics

The word 'Probiotc', derived from the Greek meaning "for life" has several different meanings over the years. It is believed that the beneficial organisms could balance the intestinal environment, prevent the growth of pathogenic bacteria and as a consequence improve health and prolong life. The administration of beneficial organisms to animals started in the 1920s and the name probiotic was introduced during 1972 where the production of bacterial feed supplements began on commercial scale.

An effective probiotic is required to operate under a variety of different environmental conditions and to survive in many different forms. It should therefore have the following characteristics; 1.It should be capable of being prepared as viable product on industrial scale; 2. It should remain stable and viable for a long period under storage and field condition; 3. It should have the ability to survive in the intestine; 4. It must produce a beneficial effect on the host animal; 5. It must able to alter the intestinal flora preventing the proliferation of the pathogen in the intestine thereby as an overall effect. Ideal probiotic organisms must be able to give improved growth, improved utilization of food and improved health. Fundamental to the understanding of the probiotic effect is the knowledge of how the specific microorganisms used can affect other microorganisms such as those, which comprise the indigenous gut microflora or invading pathogens.

Mode of action of probiotics may be attributed either to one or combination of reasons: 1. Competition for nutrients; 2. Production of metabolites like lactic acid, other organic acid and antibacterial substances etc. and 3. Competition for association sites.

Currently available probiotic preparation contain organisms like *L. delbruecki* subsp. *bulgaricus*, *L. acidophilus*, *L. casei*, *L. fermentum*, *L. plantarum*, *L. brevis*, *L. cellobiosus*, *L. lactis* and *L. reuteri*, several species of Streptococci, *Leuconostoc*, *Pediococcus*, *Propionebacterium* and *Bacillus*. The emerging probiotic organisms are several species of yeasts, *Pseudomonas* and *Micrococcus*. As on today, all probiotic preparations available for aquaculture are the ones adopted from Veterinary Science and because of the same reason, all of them suffer several set backs at practical level. This implies that the probiotic preparation exclusively for aquaculture have to be evolved for sustainability.

Holistic approach in health management

Vaccines, immunostimulants and probiotics are three powerful prophylactic tools for protection of health of the cultured species. However, the time and mode of application of these preparations are of paramount importance as the performance depends on how the animals respond to them. The defense mechanism of aquatic animals, being poikilothermic, is very much depended on the living environment and fluctuations in the system beyond tolerable levels shall result in lesser efficiency of the prophylactic tools. Therefore it is

essential to develop package of practices by integrating bioremediation programmes and the appropriate and timely application of the above described prophylactic tools coupled with closes monitoring of the pathogens in the system for an overall health management of the cultured species. It has to be accepted that these packages shall vary from species to species and place to place.

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MANAGEMENT OF FISHH/SHELLFISH DISEASES USING IMMUNOSTIMULANTS ISOLATED FROM MARINE NATURAL PRODUCTS AND OTHER ADDITIVES

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INTRODUCTION

Application of antibiotics and other chemicals in aquaculture has its own intricate problems. For example, regular use of antibiotics in fish or shrimp hatchery or grow out system may lead to development of not only antibiotic resistant fish/shrimp bacteria, but also human bacteria. Information is still lacking on the absorption and distribution of antibiotics in fish and shrimp and persistence of residues or effects of them in the environment. Hence, promoting the holistic systems approach in managing fish/shellfish health problems needs special attention.

Considering the potential threat of diseases on the one hand and the environmental issues on the other hand, disease management aspects should concentrate on environment friendly biotechnological methods like: prophylaxis (vaccines, immunostimulants, bioremediation of environment – including the methods of administration) and disease treatment protocols.

BACTERINS/VACCINES

A vaccine could be defined as a substance that causes a specific immune response. Vaccination as a part of standard fish culture programme is relatively new although the impact of vaccination is found to be dramatic. For example, the culture of salmon in brackish water and marine environment was made possible by usage of the Vibriosis vaccine, which led to a great expansion in pen-rearing of Atlantic salmon in Norway and Chile. Several studies have been undertaken on the possibility of using live attenuated vaccines.

Considering the importance of vaccination, biotechnologists are trying to develop subunit vaccines, i.e., vaccines consisting of the major protective antigens of the pathogen. The sub-unit vaccines have evident advantages: The important advantage is that the vaccine contains only a component of the pathogen and is therefore, more chemically defined and likely to be more stable. The other advantage is that the vaccine can be produced by direct synthesis or recombinant DNA technology. Thus these vaccines may be genetically engineered to express further protective antigens from other fish pathogens and thereby yield multivalent vaccines.

Techniques in administering vaccines, bacterins and immunostimulants

The route of exposure of the immunizing antigen has a direct impact on the levels and types of protective immunity that develops. Currently four methods are commonly used to deliver antigens:

1. Injection (intraperitoneal, intramuscular or subcutaneous) induces highest levels of protection; but is very labour-intensive and stressful. Semi-automated devices, which can immunize 4000 fish per hour, have been developed which reduce the stress on fish and risk of exposure to the worker.

2. Vaccination by immersion is perhaps the most widely used method. In this method, fish are dipped for 20 seconds in a well-aerated vaccine suspension. Thus a litre of vaccine can be used to vaccinate 10,000X10g fish. Dip vaccination would be stressful, but the problem is overcome by bath vaccination where fish are vaccinated by being exposed to higher dilutions of vaccines (e.g. 1:100) for times ranging from 20 minutes to several hours. Vaccine can be added directly to hatchery troughs or transport bags.

3. Spray vaccination is another modification of direct immersion but in this method fish must be handle thus making it stressful. The level of protection, though variable has been reported to be comparable to immersion.

4. Immunization by oral route by incorporating the bacterin in the feed is a potentially useful method.

IMMUNOSTIMULANTS

Immunostimulants are substances, which elicit non-specific defense mechanisms and enhance the barrier of infections against pathogens. They are isolated from natural sources and then synthesised chemically. (Ex: cell wall preparations from bacteria, fungi, mushroom).Most of the research on immunostimulants has been directed towards treatment of cancer in humans. Immunostimulating compounds induced production of cytokine proteins like interleukins, interferon, tumor necrosis factor and colony stimulating factors. Injection of such preparations resulted in improvement of symptoms from malignant tumors and prolongation time of cancer patients.

The active principles of immunostimulatory cell wall preparations are various muramylpeptide fragments, lipopolysaccharides, lipopeptides, acyloligopeptides and specific ides composed of glucose units which are linked through b-1, 3 and b-1, 6 bonds. These glucans can exist in various structural forms, water soluble oligomers; water insoluble macromolecules and particulate matters.

In fishes, the killed mycobacteria and muramy1 dipeptide enchanced resistane of coho salmon, *Oncorhynchus kisutch* against several bacterial pathogens. Injection of the synthetic lactolyl tetra peptide FK-565, increased the phagocytic activity and non-

specific resistance of rainbow trout against *Aeromonas salmonicida* infections. Resistance of carp to infections by *Edwardsiella tarda* by activating the non-specific was achieved by administration of schizophyllan, scleroglucan and lentinar. The non-specific disease resistance in Atlantic salmon was enhanced by glucan preparation from *Saccharomyces cerevisiae*. Since then several researchers have suggested the possible use of glucans against viral infections in fish and shrimps.

In shrimps, the Wheatgerm Agglutinin (WGA), a lectin, administered as feed additive has promoted the bacterial resistance of *Penaeus orientalis*.M-Glucan (a mixture of insoluble *b*-1, 3 and b-1, 6 poly glucoses) was found as a short-term immunostimulant for the shrimp, *Penaeus monodon*. Immersion treatment with yeast beta-glucan was demonstrated to enhance growth and vibriosis resistance in tiger shrimp *P. monodon*. In the treated shrimps, the disease resistance could be correlated with enhanced phenoloxidase activity and intrahemocytic production of superoxide anion.

In shrimps the prophenoloxidase ('Propo'), the defense enzyme system, is activated by immunostimulants. The activation of 'Propo' results in recognising pathogens and providing resistance.

In fish, the non-specific defense system is activated by the immunostimulants. The first line of defense - i.e., non specific humoral defense or proteases, lysins and agglutinins in mucous cell secretion; The second line of defense provided by the mucosal lining cells and the third line of defense achieved by blood cells, especially granulocytes and monocytes which destroy microbes present in the circulation are activated. Endocytically active cells such as endothelial cells, macrophages and granulocytes in organs and tissues, which degrade microbes or microbial products, take up the final defence. The final endocytic and degradation process strongly depend on the effectiveness of reticulo endothelial system, which consist of endothelial cells, and macrophages, which line the small blood vessels (sinusoids and ellipsoids). The central cells in the production of antimicrobial substances are macrophages and granulocytes, which are activated by the immune enhancers.

Hemocytes are also activated by immunostimulants. In addition, they enhance the clotting activities and produce bactericidins. In tiger shrimp *Penaeus monodon,* increased bacterial clearance was noted after injection with glucan. The bacterial clearance ability of haemolymph drawn from the tiger shrimp *Penaeus monodon* immersed in a viable cell suspension of *Vibrio vulnificus* showed that *Vibrio* cells were largely eliminated from shrimp haemolymph within 12 h following invasion and completely undetectable at 24 h. The anti-*E. coli* activity of plasma, phenoloxidase (PO) activity, as well as the production of superoxide anion (O^2) were significantly enhanced due to administration of glucan and zymosan. Immunostimulants can promote recovery from the status of immunosuppression caused by stress. The peptidoglycan- fed black tiger shrimp exhibited a higher tolerance to dissolved oxygen, salinity and stress than those fed with the controlled diet.

The immunostimulants have several advantages:

1. Being natural products, there is no environmental hazard.
2. Unlike vaccines, which give protection to a specific pathogen, immunostimulants provide a wide range of protection against several pathogens.
3. Most of the immunostimulants can be synthesized and the problem of residual effect on shrimps or fish is not encountered.
4. Fish depend more heavily on non-specific defense mechanisms than mammals and therefore immunostimulants have a significant role in health management strategies in aquaculture.
5. When glucans were administered along with *Aeromonas hydrophila* vaccine, the response was even more enhanced, suggesting that yeast glucans have important role in disease management in warm water aquaculture.

In shrimps, three main types of circulating haemocytes have been identified and isolated by isopycnic cetrifugation on Percoll gradient. Semi granular cells respond to microbial polysaccharides such as lipopolysaccharides and B-1,3-glucans by degranulation. Since the degranulated cells attach and spread on foreign surfaces, they have an important role in encapsulation. Granular haemocytes with large granules are a repository for the prophenoloxidase (pro-PO) activating system. In crustaceans, clotting is mediated by coagulogens present in the plasma and also compartmentalized within circulating cells. The plasma factor is converted to covalently linked polymers of coafulin by Ca^{2+}-dependent transaminase whereas the cell factor is converted to a gel by a serine protease proclotting enzyme, which may be triggered, by microbial molecules such as lipopolysaccharide (LPS) and -1,3-glucans.

BIOREMEDIATION OF THE FARMING ENVIRONMENT

One of the important prophylactic measures against the disease management in aquaculture is proper water management. In culture conditions, the disease problems are linked to the stress factors arising out of inadequate physico-chemical and microbiological quality of water. Ammonia and hydrogen sulfide are two important factors of great significance to the well being of the cultivable species. As the culture progresses and biomass increases, the water quality deteriorates due to accumulation of metabolic waste of cultured organisms, decomposition of unutilized feed and decay of other biotic materials. It is reported that many of the pathogens isolated from diseased shrimp were the normal flora of the culture system, which become opportunistic pathogens. For the eco-friendly environment and disease management, the concept of 'probiotics' is gaining importance.

Presently a variety of commercial products of water additive probiotics are available. The 'probiotic organisms' work on the principle of competitive exclusion. This ecological process modifies the microbial species composition of the host and its environment. The probiotic application also acts as a "bio control", through which pathogens can be killed or reduced in number in the aquatic environment. Thus the

concept of "bioremediation" is initiated, when microbes are used to treat pollutants or waste, which break down undesirable substances.

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Table 1

List of immunostimulants with promising immunostimulatory effect in lab experiments

Immunostimulants	*Species*	Pathogen	References
Glucan	P. monodon	Vibrio	Raa et al., 1996
β 1-3 Glucan	P. monodon	WSSV	Chang *et al.*, 1999
β1-3 Glucon	Marine Shrimp	Virus	Dugger and Jory 1999
Glucan	Crustacean	Disease	Takaharhi and Itami 1997
Glucan Zymoson Heat killed Vibrio LPS Trypsin	*P.monodon* *M.rosenberigi*	*Vibriosis*	Sung *et al.*, 1998
Glucan	*P.monodon*	Vibriosis	Teunissen *et al.*, 1998
β 1-3 glucan peptido glucan	Crustacea	Disease	Takahashi *et al.*, 1995
β glucan	*P.monodon*	Microbicidal activity	Sung *et al.*, 1996
β Glucan	*P.monodon*	Vibrio	Liao *et al.*, 1996
Vibrio aliginolyticus bacterin	*P.monodon*	Bacteria	Adams,1991
Glucan	*P.monodon*	*Vibrio* vulnificus	Sung *et al.*, 1994;
Vibrio bacterin, Yeast β1-3 glucan	*P.monodon*	*Vibrio* sp.	Davaraj *et al.*,1998;
Peptidoglycan	*P.monodon*	Virus	Itami *et al* ., 1998
Lipopoly saccharide & β1-3 glucan	*Pacifastacus leniusculus*		Lee *et al.*, 2000.
Yeast β1-3 glucan Lipopoly Saccharide	*P.monodon*	White spot Baculo virus	Karunasagar *et al.*, 1996;
Vibrio bacterin	*Shrimp*	Vibrio	Horne *et al* ., 1995.
Pepidoglucan	*Shrimp*	Yellow head Baculo virus	Boonyaratpalin *et al.*, 1995.
Yeast glucan	*Shrimp*		Song and Hsieh, 1994.

β1-3 glucan	Brown Shrimp		Vergas *et al.*, 1996.
Glucan	Brown Shrimp		Hernandez *et al.*, 1996
β1-3 glucan	Crustacean		Cerenius *et al.*, 1994.
β1-3 glucan	Crustacean		Sugumaran and Nellaiappan.1991.
β1-3 glucan	Shore Crab		Smith *et al.*, 1984.
β1-3 glucan	Horse shoe crab		Soederhaell *et al.*, 1985.
Chitosan Levamisole	*Panulirus homarus*		Huxley *et al.*, 2000

Table 2

Marine natural products with promising immunostimulatory effect

Source of organism	**Experimental organism**	**Assay/inhibitory activity**
Porphyra yezoensis (Seaweed)	Murine	Phagocytic assay
Undaria pinnaftifida (Seaweed)	NS	Immunomodulatory
Ecteinascida turbinata (Tunicate)	Eel	Phagocytic assay
Haliotis discus hannai (Abalone)	Trout	Phagocytic assay NK cell assay
Hyrtiss erecta (Sponge)	NS	Immunomodulatory
Briareum exavatum (Gorgonid)	NS	Immunomodulatory
Ulva fasciata (Seaweed)	Shrimp and fish	Immunostimulatory

NS – Not Specified

BIOREMEDIATION IN AQUACULTURE SYSTEMS

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Introduction

In aquaculture systems there exist a continuous exchange of substances between the bottom sediment and the overlying water, and between the two and the reared animal body. This exchange is strongly influenced by the inputs made continuously in to the system. As a matter of fact the bottom soil conditions and water quality in ponds are very much closely interrelated. Obviously, the water quality in ponds is very much influenced by the nutrient inputs, organic matter content, primary productivity and dissolved oxygen. Any aquaculture system has a finite capacity to assimilate nutrients, organic matter and the byproducts of degradation to a level congenial for the animals to grow. Bioremediation aims at the maintenance and enhancement of this finite capacity of the pond in favour and well being of the stock. Bioremediation in principle use microorganisms to transform/ decontaminate toxic pollutants in the environment. The process has two basic methods 1. Bioaugmentation, 2. Biostimulation. One of the main concerns of bioremediation is that the degradation products, whatever it may be, should be non toxic to the stocked animals. Major advantages of bioremediation are 1. It can be done on site (*in-situ*) 2. The process does not lead to any site disruption, 3. There is every possibility for permanent waste elimination, 4. Being a biological process it will be comparatively too inexpensive and 5. Can be effectively coupled with other treatment technologies

Events in a Shellfish/Finfish grow out systems

In any finfish and shellfish grow out systems, requirement of supplementary feeding is a reality. In all such situations large part of the feed gets wasted without contributing to the growth of the animals. Bacteria degrade the organic matter and it leads to low dissolved oxygen tension in the water column during night. Besides there is accumulation of faeces and also dead phyto and zooplankton in the pond bottom. All these lead to the development of anaerobic processes in pockets. Simultaneously, as the excretory metabolites of animal and also as the product of bacterial de-amination copious ammonia also gets built up.

Microbiology of anaerobic processes

Anaerobic digestion is accomplished by microbes, which can use molecules other than oxygen as terminal electron acceptor. From the thermodynamic considerations the electron acceptors are preferred by microbes in a definite order as Oxygen, Nitrate, Iron,Sulphate, Carbon Dioxide and Organic matter. Anaerobic decomposition therefore

progresses in stages as 1.Hydrolysis, 2.Acid production, 3. Nitrate reduction/ de-nitrification, 4. Iron reduction 5. Sulphate /sulphur reduction and 6. Methane production

Hydrolysis of polysaccharide results in the generation of hexose and pentose sugars, lipids in the formation of fatty acids and glycerol and proteins in the release of aminoacids.

These monomeric substances pass through the cytoplasmic membrane in to the cell by active transport and enter in to several degradative pathways. Monosacharides enters in to Embden Meyer Hoff Parnas pathway resulting in the formation of acetic acid or ethanol, CO_2 and water. Energy is stored as ATP and NAD is the Co-enzyme often involved in hydrogen transfer reaction, which must be regenerated with coupled reactions.

It is generally agreed that the anaerobic degradation of long chain fatty acids proceeds primarily via β - oxidation in which two carbon atoms at a time are split from the chain. The sequence shows the removal of one acetate unit which combines with reduced Co-enzyme A (CoA – SH). Acetic acid can then either be liberated from Co-A in a subsequent reaction or the acetate can be transferred to other functional compounds. FAD is the prosthetic group for a wide variety of enzymes associated with the hydrogen transfer reactions.

During anaerobic decomposition of aminoacids some aminoacids may be fermented individually by pathways specific to that compound. For example from a molecule of glutamic acid pyruvic acid and a molecule of acetic acid are formed. In Stickland reaction pairs of aminoacids are fermented- one aminoacid acts as the electron donor (oxidized) while the other as the electron acceptor (reduced).

Organisms involved in the hydrolysis and acid production are *Clostridium, Acrobactor, Bacillus, Escherichia, Micrococcus, Panacolobacterium, Proteus, Pseudomonas, Sarcinia, and Streptococcus*

Reduction of Nitrate

In the absence of oxygen, for the smooth conduct of hydrolysis and acid production, nitrate plays the role of electron acceptor and gets itself reduced which can be either Dissimilatory nitrate reduction or Denitrification. During dissimilatory nitrate reduction nitrate is reduced to nitrite where nitrate serves as the electron acceptor. During reduction of nitrite to ammonia there is dumping of excess energy in the molecule by the organism. Denitrification on other hand leads to the reduction of nitrate to nitrogen through nitrous oxide where nitrate serves as the electron acceptor.

Reduction of Ferric ion (Fe^{3+}) to Ferrous ion (Fe^{2+})

When nitrate is completely used up ferric ion takes up the position of nitrate as electron acceptor and become soluble. This means that when nitrate is present the redox potential will not drop low enough for ferric ion to be reduced. Ferric ion must then be expended before hydrogen Sulphide (H_2S) is produced by reduction of sulphate.

Dissimilatory Sulphate/Sulphur reduction

Dissimilatory sulphur reducing bacteria are able to oxidize acetate completely to carbon dioxide using elemental sulphur as the electron acceptor (eg. *Disulphuromonas*). Meanwhile the sulphate reducing bacteria are capable of reducing sulfate, thiosulphate or any oxidized sulphur compounds as electron acceptors leading to the production of H_2S (eg. *Desulphovibrio*)

Methane production stage

Methane gets generated from the low molecular weight acids by methanogenic bacteria. These organisms are capable of coupling organic oxidation to reduction of CO_2. Methanogens are subdivided as 1. Hydrogenotrophic methanogens, and 2. Acetotrophic bacteria. Hydrogenotrophic methanogens utilize Hydrogen chemolithotrophically and convert CO_2 to methane. Meanwhile acetoclastic bacteria cleave acetate in to methane and CO_2

Bioremediation requirements

In any aquaculture system bioremediation should focus on the removal/ transformation of 1. Detritus, 2. Ammonia and 3. Hydrogen sulphide by way of bio-augmentation and bio-stimulation.

1. Bioremediation of detritus

It is a myth that organic matter is continuously accumulated and transferred from one crop to another. Instead, what happens is that the anaerobic conditions at the pond bottom are being generated due to accumulation of fresh organic matter during the current crop. The first option is to try for the aerobic degradation of organic matter by way of re-suspending it by using aerators/ agitators. While doing so care should be taken not to re-suspend soil particles and the subsequent disturbance of hypolimnion. Aeration by way establishing uniform water current over the bottom is good option. During the aerobic degradation ammonia gets liberated which may get accumulated to toxic level if nitrification is not set in.

It has to be remembered that oxygen cannot move down to soil as the interstitial spaces are filled with water. In this situation soil microbes revert to anaerobic respiration producing H_2S, NH_3^+, N_2, H_2 and CH_4. If the hypolimnion remains intact, the organisms in this film of oxygenated sediment in the sediment water interface oxidize the above elements and compounds and prevent their diffusion in to the water column. As the order of preference of electron acceptors next to oxygen is nitrate the best option is to supply nitrate to the soil as the electron acceptor and stop the progress of rest of the anaerobic process. In such a situation the end product will be N_2, a harmless gas. Another advantage of the situation is that the phosphorus from water column gets precipitates from water columns as ferric phosphate. This will help in controlling

phytoplankton bloom. The main concern of nitrate supplementation is not to allow the redox potential falls below – 150mV

As part of bio-augmentation specific microorganism can be used for the faster degradation of the detritus. The organisms thus selected must be able to degrade organic matter faster than the native flora. They must be highly versatile to get adapted to the dynamic pond bottom and must be able to use NO_3 as the electron acceptor in the event of oxygen depletion. Two species of *Bacillus* such as *B.subtilus* and *B.lichiniformis* are best candidate species for bioremediation. They can be mass-produced, mixed with sand or clay and broadcasted for depositing in pond bottom. As a matter of caution care must be taken to avoid heavy oxygen demand, which may arise during application. As a matter of fact regular application will be required for sustained detritus removal.

2. Bioremediation of Ammonia

Biological nitrification is a natural process carried out mainly by chemolithotrophic bacteria, which derive energy by oxidizing NH^+ to NO_2 and NO_3. CO_2 serves as the carbon source. Nitrifiers are slow growers with a generation time of 10 to 60 hours. Temperature optima range from 28-30^{0}C and pH 7.6 to 8.5. Visible, long wavelength and UV light are lethal due to photo oxidation of Cytochrome C

The bioremediation protocol includes mass production of appropriate nitrifying cultures/ consortia, application in water at sunset, aeration during night, maintenance of good phytoplankton bloom and monitoring NH_3 and NO_2 level.

2. Bioremediation of Hydrogen Sulphide

Hydrogen sulphide is toxic to aquatic animals as it binds with enzymes and blocks the oxidative process. It subsequently reduces the oxygen carrying capacity. Hydrogen Sulphide is soluble to waters in 4000ppm. Aeration can take care of H_2S only if the concentration is less than 2.0ppm. Gas absorbents like zeolite can give only temporary relief. Only option is to adopt biological method.

Anoxyphotobacteria splits H_2S into elementary sulphur and hydrogen ion during photosynthesis. They grow at sediment water interface in regions where oxygen tension is < 0.1mg/L and visible light of long wavelengths, not absorbed by phytoplankton and microalgae reach. Anoxyphotobacteria have three families such as Rhodospirillaceae, Chromataceae and Chlorobiaceae. Rhodospirillaceae utilize organic matter as source of hydrogen (non-sulphur photobacteria). They are efficient mineraliser at pond bottom as they grow in both anaerobic and aerobic conditions even in dark without utilizing solar energy. Chromataceae are purple sulphur bacteria, which hold sulphur particles in cells. Chlorobiaceae are green sulphur bacteria which precipitates sulphur particles out.

These organisms can be mass cultured and applied at pond bottom. Being autotrophic and photosynthetic mass cultures become less expensive. Cells can be adsorbed on to sand grains and broadcasted.

Closed culture systems - a way out

Effective implementation of the above bioremediation programmes will lead to successful development of closed culture systems. Such systems are ideal in the scene that the entry of potential pathogens from the surrounding water bodies can be prevented. Besides, the impact of culture system on the outside aquatic environment also would be marginal as the residue of management chemicals used in the culture system also are not allowed to go out, instead sufficient time is given for their bio-degradation. Bioremediation coupled with the introduction of closed culture system will be the next phase of development in the aquaculture industry world over.

CFDDM Technology of closed system shrimp culture

Centre for Fish Disease Diagnosis and Management, CUSAT has developed recently and demonstrated successfully its first generation 'closed system shrimp culture' by incorporating the principles of bioremediation. It is designated as the first generation closed system technology due to the fact that human intervention in the process has been made possible only in the realm of detritus management by bioaugmentation and nitrification and H_2S removal have been stimulated indirectly. On completion of the currently undertaken research programmes in bioremediation by this Centre the second generation closed system shrimp culture shall be made available for practicing.

In the detritus management programme 'Detrodigest' an efficient bioaugmentor developed by the Centre is applied regularly to the pond starting from the phase of pond preparation to harvest once in 10 days. This preparation consists of highly degradative indigenous bacteria, which are capable to multiply rapidly in the pond bottom and wipe out the uneaten food, faecal matter and dead phyto and zooplankton. As a consequence rapid mineralization takes place leading to perpetual plankton bloom. Finer details of the process is available with the Centre and a booklet consolidating the technology has been published in Malayalam for the benefit of farmers of the state of Kerala. Besides the Centre offers technical assistance to shrimp farmers in the implementation of the technology in toto.

Further Readings

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TRUSS NETWORK ANALYSIS FOR FISH GENETIC STOCK DISCRIMINATION

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Introduction

Groups of potentially interbreeding natural populations, which are reproductively isolated from other such groups, is referred to as an animal species. Both genotypic and phenotypic homogeneity among groups belonging to the same species are seldom seen due to factors like environmental differences, isolation by distance and natural selection. These distinctive groups are known as races and referred to as stocks in the case of fish species.

Stock

- A self-sustaining group of individuals sharing a common unrestricted gene pool.
- Genetically distinct populations within a species, which are unique biological entities.
- It is a panmictic sub unit of a species that is generally in Hardy Weinberg equilibrium.

Stock variability is important to a species for continued successful reproduction and adaptation. Fishery biologists are interested in stocks to understand the spatial and temporal dynamics of stock differentiation and to use this information for conservation and management of the species. In fisheries it is important to identify the geographical distribution and genetic characteristics of stocks. The two popular methods of stock identification are

i. Identification based on gene frequencies through Protein gel electrophoretic studies.
ii. Identification based on morphometric studies.

Morphometrics

Morphology is a primary and direct means by which organisms interact with environment. In experimental biology it is useful to know whether two populations of organisms/organs have the same typical body form to indicate

- size allometry.
- shape changes accompanying size increase over the life span.
- to characterize the difference between sexes.
- response of form to therapeutic intervention.
- response to environmental variation etc.

- Morphometrics is the study of the geometrical form of organisms, which combines themes from ***biology***, ***geometry*** and ***statistics***. Here the ***geometric form*** of organisms is analysed.

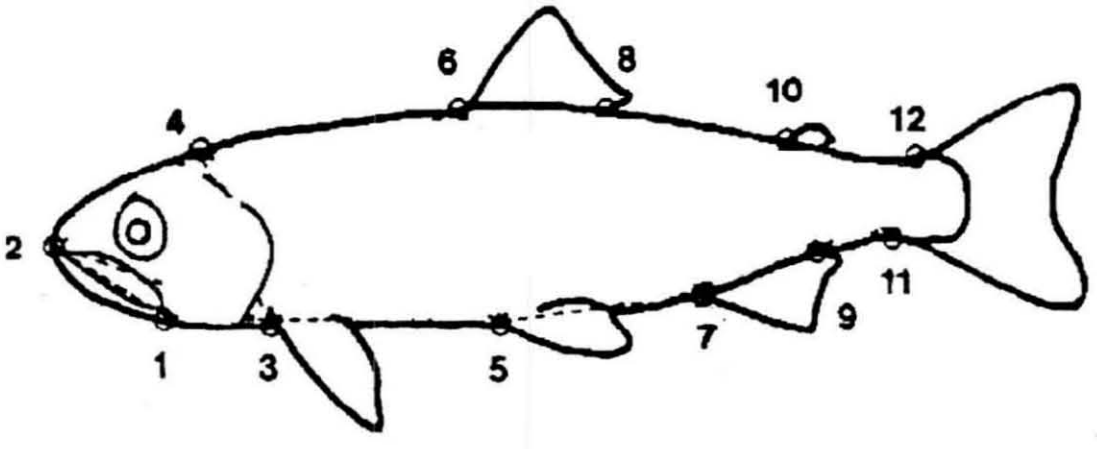
- Morphometric studies require information from ***biological homology*** and ***geometric location***.

- Biological homology is a spatial or developmental correspondence among definable structures or parts. (E.g. separate bones, nerves, and muscles). In the context of morphometrics it becomes a correspondence not of parts to parts but of points to points called a homology mapping.

- In morphometrics we study the [illegible] of biological [illegible]. The variation in the relative locations of sets of homologous points over a sample of forms.

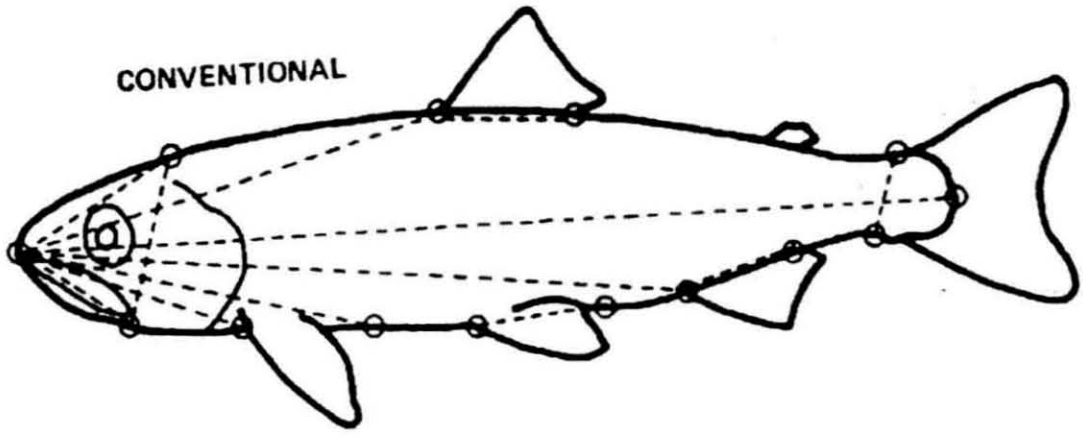
- The map of the organism is normally sampled at small number of discrete points called ***landmarks***.

Landmarks



Landmarks are defined intrinsically in terms of the anatomy in their vicinity. These are points pointed out by biologists when we talk about form of an organism. Some of the landmarks are located by juxtaposition of different identifiable structures (E.g. Anterior fin base and posterior fin base delimit the fin upon the body outline). Other landmarks are located by geometric properties (E.g. Point where the curvature of an edge is maximum).

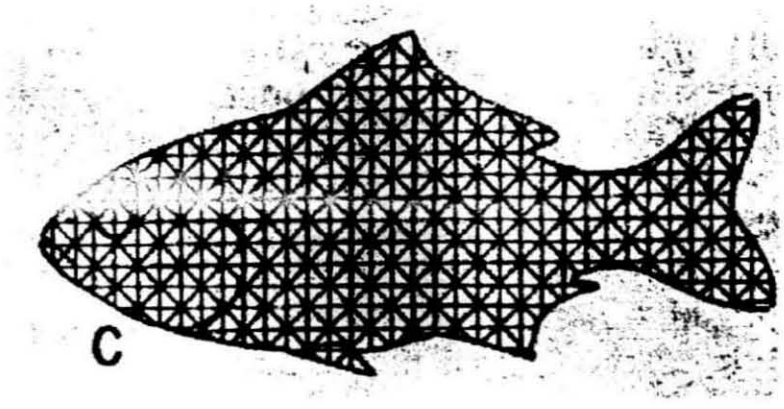
Truss Network Analysis: In systematics the interest is often in quantifying differences in form among different species or conspecific populations. When these are studied using conventional measurements (shown below) the amount of information available for analysis are repetitious and lack variation in oblique directions.



There are several biases and weaknesses inherent in traditional character set used to study stock differences in systematics.

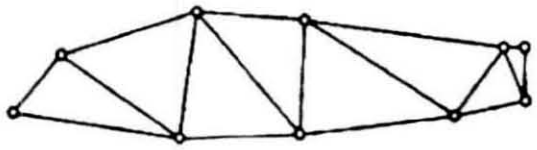
- They tend to be in one direction only (longitudinal) lacking information of depth and breadth.
- Coverage is highly uneven both by region and orientation
- Some landmarks like tip of the snout and posterior end of vertebral column are used repeatedly.
- Many landmarks are external rather than anatomical and their placement may not be homologous placement may not be homologous from form to form.
- Many measurements extend over much of the body.
- When measurements are taken on soft-bodied organisms, the amount of distortion due to preservation cannot be easily estimated.

The most ideal measurements, which overcome these problems, is as in the picture down below.

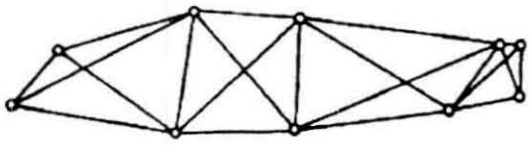


Alternative types of measurements are:

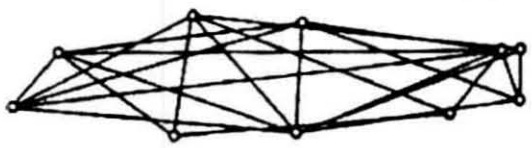
A. TRIANGULATION 2n-3 distances



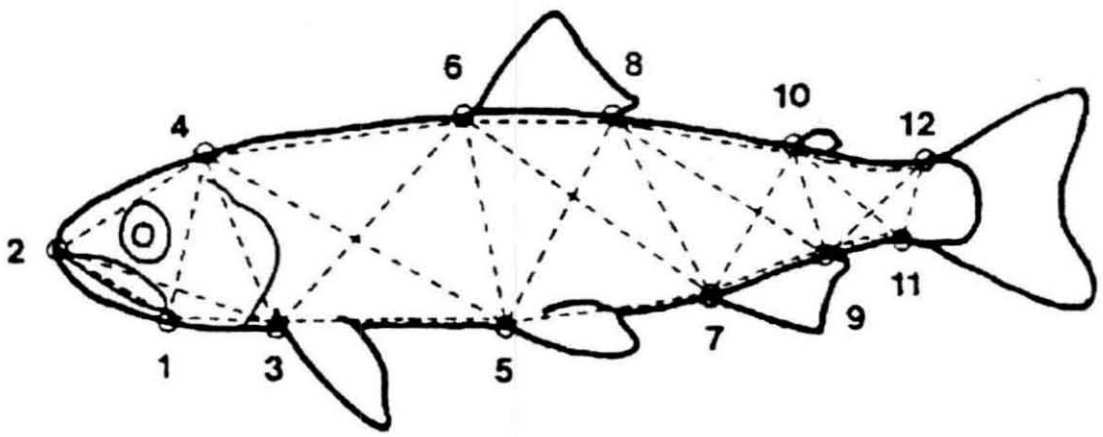
B. TRUSS 5n/2 - 4 distances



C. GLOBAL REDUNDANCY 3(n-2) distances



Truss is a geometric protocol for character selection, which largely overcomes the disadvantages of conventional data sets, and it leads to certain style of analysis. In *truss* system, homologous landmarks on the boundary of the form are divided into two tiers and paired. The distance measures connect these landmarks into an over determinate truss network which is a series of quadrilaterals each having internal diagonals. Each quadrilateral shares one side with each succeeding and preceding quadrilaterals (see figure below).



The following are the properties of a truss network measurements.

- It enforces systematic coverage across the form

- It exhaustively and redundantly archives the form
- The degree of measurement error in data can be measured and corrected
- Forms may be standardized to one or more common reference sizes by representing measured distances on some composite measure of body size and reconstructing the form using the distance values predicted at some standard body size.
- Principal components can be given geometrical interpretations. Component scores are measures of configuration while loadings are descriptors of shape change.
- Composite mapped forms are suitable for biorthogonal analysis of shape differences between forms.

Collection of Truss Measurements Data

Different Methods of collection of truss measurements data are:

- Position the specimen on light plastic in the field and take photograph with a scale in the frame. Prepare slide and project on to a digitizing tablet attached to a graphic terminal. With appropriate software locate the landmarks using the cross hairs of mouse and store the co-ordinates of landmarks.

- Place the specimen on water-resistant paper and tease the body posture and fins into a natural position. Around the outline of the form identify the landmarks. Record each landmark by making a hole in the water resistant paper with a dissecting needle. *Transfer the co-ordinates of landmarks by placing the paper on a digitizing pad and depressing the attached digitizing stylus into each hole.*

- Make the truss measurements using digital calipers connected to a Polycorder data logger. Using scanner and digitizer connected to a computer, images of specimens can be digitized and stored. With the help of an image processing software the landmarks can be identified and the truss measurements can be made.

Data Analysis

Classification problems exist in numerical taxonomy in biology and many other branches of Science. The interest here is to classify objects into one of many existing classes and is based on [illegible] taken on a set of [illegible] (called variables). Hence classification is [illegible] two to [illegible].

- We have multiple measurements data from a number of individuals belonging to known groups. Also we have data collected on individuals whose group membership is not known and is to be determined using the measurements made on them. This problem in statistical terminology comes under Discriminant Analysis.
- Another type is the case when the groups are them selves unknown and a primary purpose of the analysis is to find groups so that those belonging to same group are similar than those belonging to different groups. This in statistics come under the heading of cluster analysis or pattern recognition.

Cluster Analysis: This involves the search through multivariate data for observations that are similar enough to each other to be usefully identified as part of a common cluster. Clusters consist of observations that are close together and that the clusters themselves are separated. If each observation is associated with only one cluster, then the clusters form a partition of the data. Finding the partition into clusters is not always easy. There are numerous methods for clustering. Some methods of making clusters starts with models like mixture models of clusters. Examples of application of cluster analysis are studying genetic diversity within and between populations of and endangered fish species, clustering species of bees into higher-level taxonomic groups, developing clusters of patients based on physiological variables, constructing a speaker-independent word recognition system etc. Numerical methods of clustering with out any model can be into three major types; *hierarchical, partitioning* and *over lapping.*

Principal Component Analysis (PCA)

The objective here is to find linear combinations of the variables so that the first linear combination accounts for maximum possible variation in the data, the second linear combination accounts for the next highest possible variation and so on.

- PC analysis produces another set of variables that are linear combinations of the original variables. The new set will have the property that they will be mutually uncorrelated (orthogonal) and by considering few of them we will be able to explain a major portion of the variability in the population.
- If there are only a few clusters, the leading principal axes will tend to pick projections with good separations.
- PC analysis tend to act as a variation reducing technique relegating most of the random noise to the trailing components and collecting the systematic structure into the leading ones.

In principal component analysis we have a sample of observations taken on a set of variables and the objective is to find linear combinations of the variables so that the first linear combination accounts for maximum possible variation in the data, the second linear combination accounts for the next highest possible variation and so on. By this we get another set of transformed variables, which are linear combinations of the original variables and they, new set will have the property that by considering few of them we will be able to explain a major portion of the variability in the population. The approach in principal component analysis is to reduce dimensions by calculating the eigen values and eigen vectors of the covariance or correlation matrix and project the data orthogonally into the space spanned by the eigen vectors belonging to the largest eigen values. These projections are interesting due to the following reasons

- If projection is an aggregate of several clusters, then these can become individually visible only if the separation between clusters is larger than the internal scatter of the clusters. Thus, if there are only a few clusters, the leading principal axes will tend to pick projections with good separations.
- It tend to act as a variation reducing technique relegating most of the random noise to the trailing components and collecting the systematic structure into the leading ones.

Suppose that we have measurements on k variables $x_1, x_2, \text{؟}, x_k$ made on n individuals. Then we have $n \times k$ matrix of data and we can work out means for these variables which we can treat as a mean vector of length k. Also we can compute the variance covariance matrix **S** matrix using this data set. This matrix will be then used to compute the k principal components, say $z_i = a_{1i}x_1 + a_{2i}x_2 + \text{؟} + a_{ki}x_k$ for $i = 1,2, \text{؟}, k$ and the amount of variation explained by each of them will be available as $\lambda_1, \lambda_2, \text{؟}, \lambda_k$ where $\lambda_1 \geq \lambda_2 \geq \text{؟} \geq \lambda_k$.

In the analysis of multivariate data collected through truss network measurements the concept is that size and ***shape*** are the two factors, which account for the association among the distance measures. *Size* is not considered as a single variable but as a factor, which is obtained as a linear combination of the distance measures. *Shape* is considered as the geometry of the organism after information about position, scale and orientation has been removed. The *shape* discriminator should be independent of size, for it to be free from the effect of growth. Principal component (PC) analysis, which does not require any prior information about groups, is used in the analysis of truss data. A logarithmic transformation is first applied to the measurements before performing the PC analysis to reduce variance due to size variation and also because according to an allometric model diverse distance measures relate loglinearly in a homogeneous population. The first component factor of the PC analysis is then interpreted as size component (which is not fully free from shape) and subsequent component factors are designated as shape variable (not fully free from size). Then a plot of the first principal component scores against the second principal component scores will more of less show clustering for different groups. The percentage of variation explained by these two factors also should be considered before making conclusions.

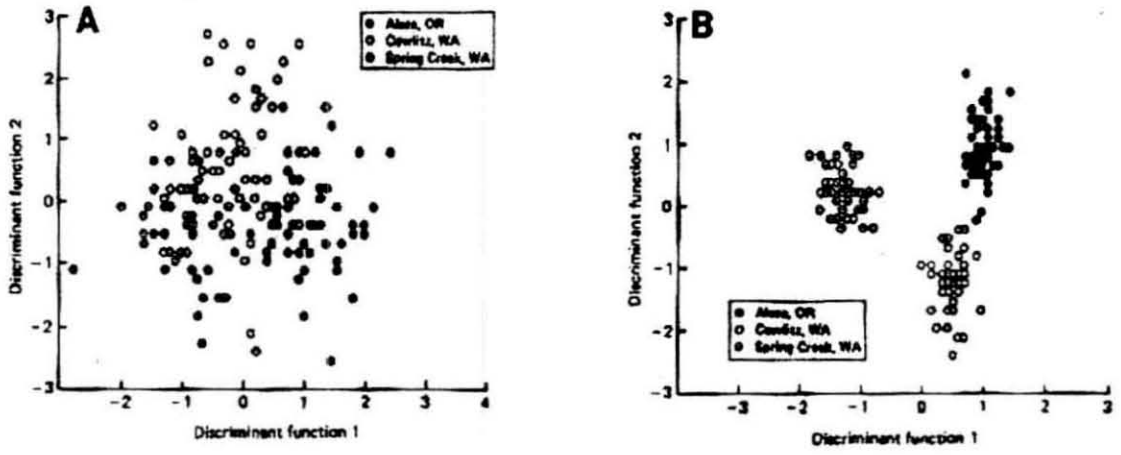
[illegible] principal component analysis of mean adjusted data by group to remove size influences from PC scores and vectors.

Steps:

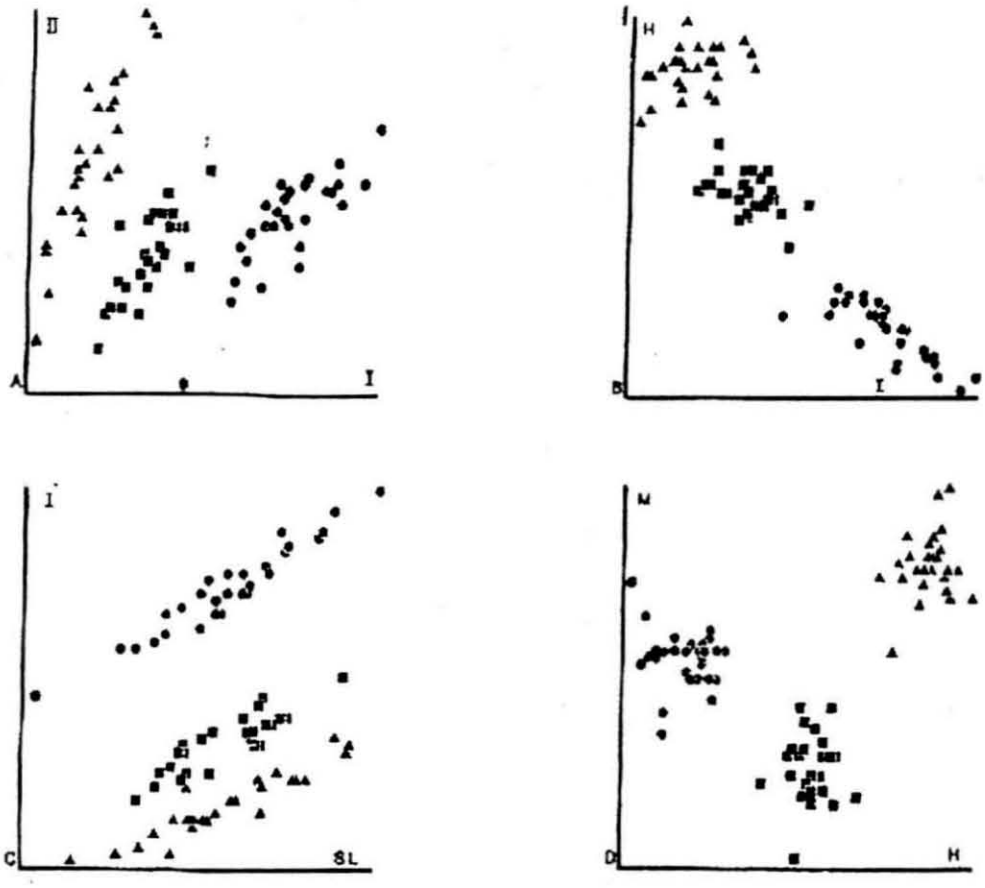
1. Transform the truss measurements data using logarithms. (According to the allometric model diverse distance measures relate loglinearly in a homogenous population).
2. Using the pooled covariance matrix ***Q*** compute the PC scores by evaluating the egen structure of matrix ***Q***.

3. From the scatter plot of the first two PC scores (say *PCI* and *PCII*) identify clusters associated with size and shape differences among populations.
4. Compute the covariance matrix Q' adjusted to zero mean for each of the identified clusters compute the PC scores by evaluating the egen structure of Q'. The first PC score, say S, will then be a within group size component.
5. Adjust the first two PC scores from the original analysis based on Q to zero mean for each of the identified clusters, say PCI_z and $PCII_z$.
6. Express the confounding of size component S with the second PC by regressing $PCII_z$ on S and denote the slope by α.
7. Estimate the portion $\hat{S}$ of S that lies in the plane of PCI_z and $PCII_z$ from a multiple regression of S on PCI_z and $PCII_z$ to yield the regression coefficients β_1 and β_2.
8. The shape discriminator H, known as the sheared factor II is then computed as, $H = -\alpha\beta_1 PCI + (1-\alpha\beta_2) PCII$. This will be uncorrelated with intracluster size and retains all discriminatory power original PC scores.

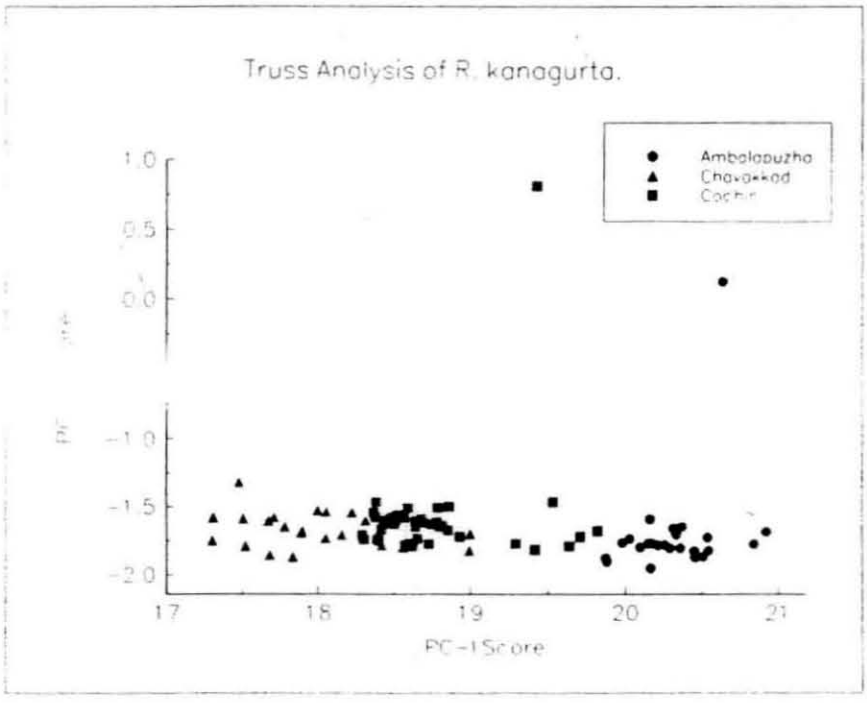
Illustrative Examples:



Scatter plot of scores based on A) conventional measurements and B) truss network measurements for chinook salmon from three locations.



Scatter plot of scores for three species of minnows A) PCI and PCII scores B) Sheared PCII against PCI C) PCI against standard length D) Sheared PCII against PC scores computed for meristic variables.



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ENZYMES FROM MARINE ORGANISMS

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Introduction

Major targets of modern enzyme technology continue to be preservation of foods and food components, efficient use of raw materials, improvement of food quality such as texture and taste, manufacture of dietetic foods, eliminating anti-nutritive substances from certain nutritional raw materials, utilization of raw materials for preparation of animal feed, and optimization of process to reduce process costs. Enzymes are used in several industries including pharmaceuticals, food, starch, laundry, detergents, textile, leather, bioremediation and industrial catalysis, organic synthesis and transformation of compounds. Enzymes are used by a plethora of industries as cost -effective and environmentally sensitive substitutes for chemical processing. Examples include those used in detergent formulations, in food processing, for the production of pharmaceuticals, for processing textiles, wood pulp and paper, and for the production of fine and specialty chemicals. hey are finding applications as research tools in biotechnology and molecular biology. As such the worldwide enzyme market is estimated at $2 billion and is expected to expand rapidly in the new millennium.

Several industrial applications demand enzymes that are stable at extremes of temperature, pH, and salt concentration. It is anticipated that extremophiles may yield novel enzymes for these purposes. For instance, the deep seabed provides many extremophiles, and consequently may be of interest to companies involved in developing enzymes for this sector. In spite of the fact that several industrial enzymes are derived from terrestrial sources, marine organisms are yet to be exploited to its full potential and consequently warrant immediate attention for industrial exploitation. Marine environment, which encompass about 71 percent of the earth's surface, is not only rich with biodiversity but also a vast resource for potential novel enzymes of useful applications. With the advent of biotechnology, enzyme engineering and introduction of other innovative technologies there is plenty of scope for efficient management of our rich marine microbial biodiversity towards deriving novel enzymes could also be recovered from marine microorganisms and efficiently exploited not only as a cost effective biocatalyst but also as a ecofriendly reagent in the coming years.

Enzymes from marine microorganisms

Bacteria and fungi from marine environments secrete different enzymes based on their habitat and their ecological functions. Marine microbial enzymes have become the focal point of interest now since 1980, and several enzymes have drawn the attention of

- There is a tendency particularly in recent years, to couple basic science as closely as possible with technology.
- More technology means more money, the country with better technology will have less unemployment, so more technology is better for everybody.
- This tendency to couple basic research with technology is very evident in genetics and genetic engineering.

Principal ethical issues created by modern Biology

- The major ethical issues arising from recent advances in biological science are:
- normally eat (eg: pig genes in to sheep) would offend jews and muslims
- Transfer of animal genes in to food plants that may be of particular concern to some vegetarians
- Use of organisms containing human genes as animal feed (eg: yeast modified to produce human proteins of pharmaceutical value and the spent yeast then used as animal feed)
- Foods or food ingredients derived from GMOs must be considered to be as safe as ,or safer than , their traditional counter parts before they be recommended as safe
- The safty of the human food supply is based on the concept that there should be a reasonable certainty that no harm will result from its consumption . Increased pathogenicity of micro-organisms or microbial ability to destroy essential raw materials are often cited as potential problems of GMOs
- Moving out in increasing numbers of recombinant plants from research laboratories and the containment green houses and test-plots to the fields and green houses of the farmer and large commercial horticulture grower.
- Recombinant microbes are now being considered for deliberated release in to environment for biological control, inoculants in agriculture, live vaccines , bioremediation, bakers and the brewers yeast .
- Unpredictable and perhaps catastrophic consequences – could upset the balance of nature or that foreign DNA in the new microoraganism could alter its metabolic activity in unpredictable and undesirable ways.
- To discuss "Ethical and Ecological Aspects of Intellectual Property Rights in the Context of Genetic Engineering and Biotechnology" in a meaningful way is as impossible as to analyze the benefits and risks of genetic engineering and biotechnology in general.
- One would have to make reference to health, agricultural and industrial as well as environmental problems, look at things from a biological, economic and social as well as cultural point of view and, last but not least, discuss every issue involved in the context of industrialized and developing countries and against background of of disparate value premises

BIOETHICS AND GENETIC ENGINEERING

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Ethics and Bioethics

- To be or not to be Good ?
- Regulating an action in the best interest of a large population of a given species of life
- Towards conservation of life, environment and biodiversity?
- Towards sustainable utilization of living and non living resources available in the planet earth?
- The moral obligation of scientists is to inform the general public of the possible ways that scientific knowledge can be used and misused.

The first ethical imperative of a responsible citizen is to be informed about the possibilities opened by science and technology and their potential dangers

- The second ethical imperative is to participate in decision making in an informed way , with personal action or political influence, in her/his own city and in the world.
- All human beings share the same boat, our planet
- New science brings new technology
- Scientific knowledge /New technology can be used in good or in bad ways. Example use of atomic bomb on Hiroshima and Nagasaki
- New science and its resulting technology therefore create new ethical problems
- Genetic engineering and biotechnology1 are considered to be amongst the most powerful and economically promising technological means for use in many areas.

Principal ethical issues created by modern Biology

"The tools of molecular Biology have enormous potential for both good and evil. Lurking behind every genetic dream come true is a possible brave new world nightmare"- Philip Elmer – Dewitt, Time, March 20th 1989.

- With knowledge of genetics we have the dilemma
- By cloning and sequencing human genes it is possible to make medical products or to cure by gene therapy
- By cloning and sequencing the very same genes it is possible to advise potential carriers of defective genes
- By cloning and sequencing the very same genes it is possible to discriminate, stigmatize, and make those who have the bad luck to carry defective genes poorerpotential carriers of defective genes

the microbial prospectors and few enzymes were isolated from seawater and marine sediments, purified and characterized for their properties and applications. Interestingly the enzymes reported from marine environments belonged to one or more of the major classes of enzymes viz: Oxidoreductase, Transferases, Hydrolases, Lyases, Isomerases and Ligases.

Novel enzymes

The modern tools of biotechnology are enzymes that are capable of carrying out very specific molecular tasks, usually related to the modification of DNA or RNA, for the creation of genetically modified organisms or for diagnostic procedures. These enzymes go by a variety of names, including restriction endonucleases, RNA and DNA polymerases, DNA ligases, alkaline phosphatases, kinases, reverse transcriptases, and so on. The market for these enzymes has been growing.

Although at global level, marine microorganisms are becoming sources for novel enzyme tools required for new innovations in biotechnology, the Indian scenario does not indicate any activity in this direction. India with its vast marine environments is a treasure of novel enzymes worth several millions dollars. If appropriately harnessed, these marine organisms would contribute significantly.

Enzymes from Marine animals

Ca-Mg-dependent endonucleases from embryos of the sea urchin, *Strongylocentrotus intermedius*, and hepatopancreas of the crab, *Paralithodes camtschatica*, were the most promising among the DNAases studied. These enzymes are representatives of a group of eucaryotic endonucleases capable of cleaving double stranded DNA preferentially as well as of recognizing conformational peculiarities of the sugar-phosphate backbone of natural DNA and synthetic polynuc-leotides.

These DNAases are stable at high temperatures and in the presence of detergents. All these unique properties make these enzymes widely used as tools for structural studies of DNA and for obtaining nucleotides and oligonucleotides.

DNA-polymerases from differentiating sea urchin embryos as well as thymidine and thymidylatekinases from the gonads of sea urchins and molluscs belong to another group of enzymes of nucleic acid metabolism. They resemble corresponding enzymes of mammals and humans in their properties. Such enzymes were utilized for screening of new marine secondary metabolites specifically inhibiting biosynthesis of DNA in cells invaded by tumours or viruses.

Extremozymes

Standard enzymes stop working when exposed to heat or other extremes, and so manufacturers that rely on them must often take special steps to protect the proteins during reactions or storage. By remaining active when other enzymes would fail, extremozymes can potentially eliminate the need for those added steps, thereby

increasing efficiency and reducing costs. They can also form the basis of entirely new enzyme-based processes.

Extremophiles are the primary source of extremozymes that are active at extreme conditions of life. Some extremophiles have been known for more than 40 years. Harsh marine environments, such as deep-ocean hydrothermal vents, polar oceans, and extremely saline bodies of water, have yielded valuable extremophile microorganisms. Further, the deep-sea is an extreme environment with especially high hydrostatic pressure and low temperature, and some bacteria and fungi have been isolated from deep-sea mud and from benthic organisms such as amphipods and sea cucumbers in the bathypelagic zone.

The hunt for extremozymes has been fueled in the past several years by industry's realization that the "survival kits" possessed by extremophiles can potentially serve in an array of applications. Of particular interest are the enzymes that help extremophiles to function in markedly hot, cold, acidic, basic, pressurized, saline or mineral rich environments. Such hardiness means "extremozymes" (enzymes from extremophiles) might function in hotter, more high-pressure manufacturing conditions compared to current industrial enzymes. As a result, extremozymes could speed up or maximize reactions used to make food, detergents, and drugs; remediate toxic waste; and drill for oil.

Enzymes from extremophilic microorganisms that are adapted to living in extreme environments are of particular interest to biotechnologists seeking new research tools. Thus, discovery of extremophiles opens up new opportunities for the development of enzymes having extraordinary catalytic capabilities. In nature, these peculiar enzymes fuel microbes that live in scalding sea vents, Hot Springs, and other adverse locales. In the hot springs of Yellowstone National Park alone, researchers have discovered more than 30 extremophile species, half of which have known biotech potential for processing starch, bleaching paper, or producing ethanol. Each organism may offer novel enzymes as well as insight into the evolution of life on earth.

Potential applications of acid-tolerant extremozymes range from catalysts for the synthesis of compounds in acidic solution to additives for animal feed, which are intended to work in the stomach of animals. The enzymes that are selected are ones that microbe normally secretes into the environment to break food into pieces suitable for ingestion. When added to feed, the enzymes improve the digestibility of inexpensive grains, thereby avoiding the need for more expensive food.

Alkalophiles live in soils laden with carbonate and in so-called soda lakes, such as those found in Egypt, the Rift Valley of Africa and the western U.S. Above a pH of eight or so, certain molecules, notably those made of RNA, break down. Consequently,

alkalophiles, like acidophiles, maintain neutrality in their interior, and their extremozymes are located on or near the cell surface and in external secretions.

Cold adapted enzymes

Cold-loving organisms have attracted manufacturers who need enzymes that work at refrigerator temperatures such as food processors (whose products often require cold temperatures to avoid spoilage), makers of fragrances (which evaporate at high temperatures) and producers of cold-wash laundry detergents.

Cold adapted enzymes tend to have fewer salt links, reduced aromatic interactions within the hydrophobic core, and reduction in the number of proline and arginine residues, a reduction in the hydrophobicity of the enzyme and an increase in the number of interactions between the enzyme surface and the solvent. It appears that the molecular adaptation resides in a weakening of the intramolecular interactions, and in some cases in an increase of the interaction with the solvent, leading to more flexible molecular edifices capable of performing at a lower energy cost. The structural bases of cold adaptation stand in almost direct contrast to the structural changes such as hydrophobic interactions, salt bridges and hydrogen bonds, that increase the thermal stability of thermophilic proteins.

Cold environments are actually more common than hot ones. The oceans, which maintain an average temperature of one to three degrees C (34 to 38 degrees F), make up over half the earth's surface. The vast land areas of the Arctic and Antarctic are permanently frozen or are unfrozen for only a few weeks in summer. Microbial communities populate Antarctic sea ice-ocean water that remains frozen for much of the year. These communities include photosynthetic eukarya, notably algae and diatoms, as well as a variety of bacteria. One bacterium *Polaromonas vacuolata,* is a prime representative of a psychrophile whose optimal temperature for growth is 4°C, and it finds temperatures above 12°C too warm for reproduction. A novel psychrostable metallo protease (almelysin) was produced from *Alteromonas* sp. Extracellular hydrolase e.g. protease, chitinase, glucanase, esterase, lipase, phospholipase and DNA-degrading enzyme have been obtained from marine bacteria isolated from different sites in permanently cold arctic and antarctic habitats. Some of the cold adapted enzymes from bacteria include alpha amylase from *Alteromonas haloplanctis,* isocitrate dehydrogenase and lipase from *Vibrio* sp, beta lactamase from and triosepphosphate isomerase from *Moraxella* sp subtilisin from *Bacillus* sp.

Extremely heat-sensitive enzymes, useful as biotechnology research tools, allow for better control over the reaction process, since heating to destroy the enzyme can terminate reactions. Since heating a reaction mixture can also affect the products of the reaction, use of heat -sensitive enzymes allows scientists to stop the reactions at lower temperatures. Heat-sensitive enzymes are derived from cryophiles, organisms living in cold environments.

Alkaline enzymes

Enzymes that are active at high alkaline pH are termed as alkaline enzymes and such enzymes are produced mainly by alkalophiles (AKs), which have made a great impact in industrial applications. Alkaline protease has industrial cleansing applications including application in standard and non-phosphate surfactant and for contact lens cleaning. Another important application is the industrial production of cyclodextrin with alkaline cyclomaltodextrin-glucanotransferase (EC-2.4.1.19). This enzyme reduced the production cost and paved the way for its use in large quantities in foodstuffs, chemicals and pharmaceuticals. Besides these applications, there are other possible applications in food and waste treatment industries.

To work effectively, detergents must be able to cope with stains from food and other sources of grease. These jobs are best accomplished by such enzymes as proteases (protein degraders) and lipases (grease degraders). Yet laundry detergents tend to be highly alkaline and thus destructive to standard proteases and lipases. Alkalophilic versions of those enzymes can solve the problem, and several enzymes that can operate efficiently in heat or cold are now in use or being developed. Alkalophilic extremozymes are also poised to replace standard enzymes wielded to produce the stonewashed look in denim fabric. As if they were rocks pounding on denim, certain enzymes soften and fade fabric by degrading cellulose and releasing dyes. Biological surfactants contain alkaline enzymes, such as alkaline cellulases (EC-3.2.1.4) and/or alkaline proteases that have been produced from AKs.

Some of the alkaline enzymes known from marine environments include thermo stable alkaline phosphatase (EC-3.1.3.1) with a wide pH range, and endo-1,3-beta-D-glucanase (EC-3.2.1.39) from *Alteromonas macleodii* KMM162; alkaline phosphatase (EC-3.1.3.1) from the marine *Alteromonas* sp. KMM 518; heat-labile alkaline phosphatase from a Gram-negative Antarctic marine bacterium; alkaline metallo endopeptidase from a marine *Vibrio* sp. NUF-BPP1; and alkaline protease from marine shipworm bacterium.

Halophilic and halo tolerant enzymes

A number of exoenzymes produced by some halobacteria have optimal activity at high salinities and could therefore be used in many harsh industrial processes where the concentrated salt solutions used would otherwise inhibit many enzymatic conversions. Intracellular concentrations of salt for extreme halophiles is higher than 3.5 M, but this is mostly KCl not NaCl. Halophilic enzymes are therefore uniquely adapted to function in conditions with low water potential. Salt-tolerant enzymes do have application in enhancing the amount of crude extracted from oil wells, through the process of incorporating the enzymes into extraction procedures.

Thermostable enzymes

Due to the high water pressures at the depth at which hydrothermal vents are found, water temperatures can exceed that of the boiling point at sea level. This environment has given rise to some of the most unusual microorganisms on the planet, able to grow at temperatures exceeding 100°C. Enzymes isolated from hyperthermophiles show a corresponding tolerance for high temperatures.

Thermophilic microbes in the deep-sea thermal vents and hyperthermophilic bacteria that grow at 80-100°C are a potential source of highly specific and extremely thermostable enzymes. Several heat-loving extremozymes resemble their heat-intolerant counterparts in structure but appear to contain more of the ionic bonds and other internal forces that help to stabilize all enzymes. Whatever the reason for their greater activity in extreme conditions, enzymes derived from thermophilic microbes have begun to make impressive inroads in industry. A different heat-loving extremozyme in commercial use has increased the efficiency with which compounds called cyclodextrins are produced from cornstarch. Cyclodextrins help to stabilize volatile substances (such as flavorings in foods), to improve the uptake of medicines by the body, and to reduce bitterness and mask unpleasant odors in foods and medicines.

Thermus aquaticus, an unusual, heat-loving bacterium, discovered in 1965 at Yellowstone, was used in the 1980s, to get the enzyme *Taq* polymerase, which subsequently became the star of high-heat polymerase chain reaction (PCR) technology. More recently, some users of PCR have replaced the *Taq* polymerase with *Pfu* polymerase, isolated from the hyperthermophile *Pyrococcus furiosus,* "flaming fireball", which works best at 100 degrees C.

A new DNA-polymerase (EC-2.7.7.7) was isolated from *Pyrococcus furiosus*, a hyperthermophilic, anaerobic, marine archaebacterium. The new enzyme (92 kDa) was characterized with respect to thermostability, DNA-polymerase, 3'-5' and 5'-3' exonuclease, processivity, strand displacement and proofreading activities.

Enzymes as tools in Biotechnology

Enzymes that are capable of carrying out very specific molecular tasks, usually related to the modification of DNA or RNA, for the creation of genetically modified organisms or for diagnostic procedures are the modern tools of biotechnology. These enzymes go by a variety of names, including restriction endonucleases, RNA and DNA polymerases, DNA ligases, alkaline phosphatases, kinases, reverse transcriptases, and so on. The market for these enzymes has been estimated to be at least $600 million. Marine microorganisms are becoming sources for novel enzyme tools required for new innovations in biotechnology. Few attempts made by investigators are presented below.

Innovations in Enzyme Technology:

In general traditional microbial enzyme technology ventures are undergoing rapid transformation through the process of evolution of innovative technologies facilitated through techniques like molecular gene cloning, protein and enzyme engineering, metabolic engineering and immobilization of enzymnes.

Enzyme engineering

Microbiologists at the cutting edge of modern industrial enzyme technology have begun to modify extremozymes, tailoring them to meet specific demands through the process of protein/enzyme engineering. For instance, after finding an extremozyme that degrades proteins fairly efficiently at high temperatures, investigators might alter the enzyme so that it functions across a broader range of acidity and salinity.

Biologists today generally achieve such modifications in either of two ways. Practitioners of the "rational design" approach first discern the structural basis of the property of interest. Next, they alter an enzyme's gene to guarantee that the resulting catalytic protein will gain that property. Devotees of the other approach, known as directed evolution, make more or less random variations in the gene encoding a selected enzyme and, from those genes, generate thousands of different versions of the enzyme. Marine microbial enzymes could become soon the subject of such endeavors.

Immobilization technology

Immobilization of enzymes by either one or more of the available methods like gel entrapment, microencapsulation, cross linking, physical adsorption, ionic binding, or covalent binding have become the subject of investigation recently for not only rapid and efficient use of the enzymes for desired bioconversion or biotransformation or organic compounds, but also for the continuous and repeated use of the catalytic enzyme. Immobilized enzyme based bioreactors, biosensors and bioprocesses have become the order of the day in biotechnology revolution in industry, targeted drug delivery and bioremediation processes in environment. Marine microbial enzymes are just in the process of exploitation.

FUTURE PROSPECTS

Enzyme biotechnology offers several new and innovative bioprocesses for the growth of bioindustry in the new millennium. Enzymes active in nonaqueous media could be harnessed for the production of chiral compounds and the synthesis of special polymers. Enzyme mediated synthesis of aminoacids and antibiotics, recycling of food wastes and wastewater treatment, production of sweeteners such as aspartame using combined microbiological and enzymatic method, production of cyclodextrins from starch are utmost important. Production of tailored enzymes to serve as specific, process-

adapted catalysts through enzyme/protein engineering and large-scale production of enzymes by gene technology is to be expected in future years. Extremozymes can be produced through recombinant DNA technology without massive culturing of the source extremophiles. As long as one can obtain sample genes from extremophiles in nature or from small laboratory cultures, those genes can be cloned and used to make the corresponding proteins using the ordinary or "domesticated" microbes, which will often use the genes to produce unlimited, pure supplies of the enzymes. In the long run, probably, marine microbial enzyme based processes would substitute several of the current chemical processes under practice.